Improved coagulation in bleeding disorders by Non-Anticoagulant Sulfated Polysaccharides (NASP)

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Summary
Additional therapeutic options are needed for patients with bleeding disorders such as hemophilia A, hemophilia B, severe von Willebrand disease, and other rare factor deficiencies. A novel approach to improve coagulation in such clotting disorders has been identified that, paradoxically, involves heparin-like sulfated polysaccharides. Select molecules of this broad class are largely devoid of anticoagulant activity and are here denoted Non-Anticoagulant Sulfated Polysaccharides (NASPs). A mechanism involving blockade of the extrinsic pathway downregulator Tissue Factor Pathway Inhibitor (TFPI) by NASPs, was conceived as an approach for improving procoagulant behavior in hemophilic settings. A subset of NASPs, including pentosan polysulfate (PPS) and fucoidan inhibited both full-length and Kunitz 1 and 2 (K1K2) TFPI and, at concentrations from 4–500 nM, improved (i.e. accelerated) the clotting time of human hemophilia A and hemophilia B plasmas or plasma with reduced factor VII levels when tested in dilute prothrombin time (dPT) assays. Fucoidan did not reduce normal plasma APTT times implying specificity for extrinsic pathway control. Improved hemostasis in vivo was observed in mice with hemophilias A or B following low dose subcutaneous administration of PPS or fucoidan, or a combination of NASP plus factor supplement. Increased survival of factor deficient mice following a bleeding challenge was observed. Accordingly, administration of select NASP(s), via mechanism(s) not fully understood, represents a unique means of improving coagulation in bleeding disorders.

Keywords
Hemophilia, sulfated polysaccharides, fucoidan, TFPI, coagulation

Introduction
The treatment of blood clotting disorders including hemophilia (Hem) A and Hem B, severe von Willebrand disease (svWD), and severe factor VII (FVII) deficiency have typically been treated by factor replacement: factor VIII for Hem A and svWD, factor IX for Hem B, and factor VII(a) for FVII-deficiency and others (recently reviewed in [1–4]). While such therapies are often effective, characteristics limiting utility include high cost, inconvenience (i.e. intravenous administration), and neutralizing antibody generation (1–5). Since FVIIa is increasingly utilized in various bleeding disorders (3), alternative single compound procoagulant therapies devoid of aforementioned constraints and with broad application should be explored.

A general approach to improving hemostasis in individuals with bleeding disorders might be to improve the initiation of clotting by upregulating the extrinsic pathway of blood coagulation. While the intrinsic and extrinsic pathways of coagulation contribute to thrombin generation and fibrin clot formation (6), the extrinsic – or tissue factor (TF) mediated – path is critical for initiation, and contributes to propagation of coagulation in vivo (7, 8). One means for upregulating extrinsic pathway activity might be the attenuation of Tissue Factor Pathway Inhibitor (TFPI). TFPI is a Kunitz-type proteinase inhibitor of FVIIa/TF which provides tonic downregulation of extrinsic pathway activation (see [9–11] for review). Indeed, heterozygous TFPI deficiency in mice can result in exacerbation of thrombus formation (12), and TFPI gene mutation is a risk factor for thrombosis in humans (13). Regulating clotting in hemophilia via the targeting of TFPI was described by Nordfang et al. and Wunte et al. who showed that anti-TFPI antibodies could shorten the coagulation time of hemophilic plasma (14, 15) and that anti-TFPI IgG improved the bleeding time of rabbits made factor VIII-deficient (16). Hence, “inhibiting the inhibitor” would seem to be a validated procoagulant approach and we therefore sought to identify novel agents yielding such an outcome.
Sulfated polysaccharides are a class of molecules characterized by a plethora of biological activities with often favorable tolerability profiles in animals and humans. These polyanionic molecules are often derived from plant and animal tissues and encompass a broad range of subclasses including heparins, glycosaminoglycans, fucoidans, carrageenans, pentosan polysulfates, and dermatan or dextran sulfates (17). Lower molecular weight, less heterogeneous, and chemically synthesized sulfated polysaccharides have been reported and have reached various stages of drug development (18–22). Heparin-like sulfated polysaccharides exhibit differential anticoagulant activity mediated through antithrombin III and/or heparin cofactor II interactions (17). Notably, certain compounds, of natural origin or chemically modified, exhibit other biological activities at concentrations (or doses) wherein anticoagulant activity is not substantial (22–26). The consideration of certain sulfated polysaccharides as candidate molecules for therapy in bleeding disorders stemmed from recognition that heparin sulfate exhibited strong interactions with TFPI (9–11, 27). We hypothesized that specific, drug-like sulfated polysaccharides could be identified that would interact with TFPI and inhibit its activity at lower concentrations than that associated with anticoagulation. Such molecules, if identified, could potentially enhance coagulation in settings wherein clot formation is compromised, including hemophilia.

Described here are several non-anticoagulant sulfated polysaccharides (NASPs) from which a subset are identified as potent inhibitors of TFPI in physiologically-relevant clotting assays. Pentosan polysulfate and fucoidan are particularly interesting NASPs as they both inhibit TFPI and improve the clotting time of human factor VII-, VIII-, and IX-deficient plasmas. The margin between procoagulant and anticoagulant activities is at least ten-fold \textit{ex vivo} and, in hemophilic mice, NASP treatment improved hemostasis alone as well as in combination with low levels of factor (FVIII).

Materials and methods

Reagents

Heparin and modified heparins, and fucoidan were purchased from Sigma (St. Louis, MO). The source of pentosan polysulfate sodium (PPS) was from the prescription drug Elmiron (Ortho-McNeil Pharmaceuticals) adjusted for active ingredient concentration. Human plasmas were obtained from George King Biomedical (Overland Park, KS). Factors VIIa and human recombinant TFPI were from American Diagnostica (Stamford, CT) and Factor VIII was prescription ReFacto® (Wyeth Pharmaceuticals). Simplastin Excel and APTT reagent were obtained from bioMerieux (Durham, NC). Recombinant pure K1K2 TFPI (TFPI 1–160) and TFPI-depleted human plasma were prepared and validated as previously described (28, 29).

Plasma clotting assays

The APTT assay was modified from standard procedures (30, 31). Briefly, 5ul of NASP at 20x the final desired concentration dissolved in saline was incubated with 95ul plasma for 30min at room temperature. Then, 100ul of 37°C APTT reagent was added to the mixture and incubated at 37°C for 3min followed by addition of 100ul of 37°C 25mM CaCl₂ and initiation of timing in a standard fibrometer. A dilute APTT assay was modified by using a 1:300 dilution of APTT reagent in hepes/saline.

The dPT assay was similar to that previously described (14). Simplastin was diluted with saline to 1:100 or 1:300, depending on the desired control clotting time, and mixed with 25 mM CaCl₂. The plasma sample was also prewarmed to 37°C and then 75ul of each were mixed together and time to clot was measured with a fibrometer. For evaluation of NASP activity, 5ul of 20X desired final concentration NASP was preincubated with plasma at room temperature for 30min before initiating the dPT assay. To assess potential inhibition of TFPI activity by NASP, diluted rTFPI was preincubated with NASP for 5min at room temperature, plasma sample was added, and the mixture was incubated for an additional 25min followed by dPT initiation. All clotting studies were performed in duplicate and reproduced.

Animals and bleeding tests

Hem-A (homozygous for the exon 16 FVIII KO allele) were licensed from John Hopkins University, and Hem-B mice (homozygous for the exon 1–3 FIX KO) were licensed from University of North Carolina at Chapel Hill. All animal procedures were performed according to “Guide for the Care and Use of Laboratory Animals” (32) and all procedures were reviewed and approved by an institutional animal care and use committee.

Procedures utilized for the animal studies were as follows: For the PPS studies, Hem-A and Hem B male or female mice were administered PPS or saline vehicle at dose levels specified in the Results subcutaneously twice daily for 5 days. On the morning of the fifth day after dosing, the tail was clipped 1 cm from the tip, and behavior and survival were monitored for the next 20–24 hrs. In the fucoidan studies, HemA male mice received fucoidan or saline subcutaneously twice daily for 4 days. On the morning of the 5th day, mice received a doubled dose of fucoidan prior to the bleeding test. For evaluation of combination therapy potential, mice in the combo groups received an intravenous bolus in a tail vein far up near the body of 53mU/mouse, which is 1.25% of the normal level of FVIII, on the morning of the fifth day. As before, the lateral tail vein, and not the artery, was transected 2 hr later at the region corresponding to a diameter of ~2.7mm. In these fucoidan studies, the tail vein transection modification was utilized as it was found to more accurately assess hemostasis and its regulation (33). Survival and clinical observations were recorded for 20–24 hrs.

Statistical analyses

For the clotting assays, Student’s t-test was used to analyze the significance between NASP-treated samples and vehicle controls. Data from the mouse bleeding tests were studied for significance from vehicle controls (or other groups as indicated in tables) by one-way Chi-squared analysis. Nearly identical results were obtained by Fisher’s exact test.

Results

Sulfated polysaccharide anticoagulant activity

Several forms of sulfated polysaccharides including modified heparins, pentosan polysulfate, and fucoidan were obtained from...
Select sulfated polysaccharides were tested across a broad range of concentrations in a dPT assay with human Hem A plasma and compared on a molar basis to each other and to heparin. As shown in Figure 1A, heparin at concentrations exceeding 10 nM was markedly anticoagulant whereas N-acetyl heparin (NAH), N-acetyl-de-O-sulfated heparin (NA-de-O-SH), de-N-sulfated heparin (De-N-SH) showed little or no prolongation of clotting time at concentrations ≥5000 nM. Likewise, fucoidan and PPS were only weakly anticoagulant, exhibiting 50% prolongation of clotting time at concentrations approximately 10- to 100-fold higher, respectively, than heparin and are hence described as “non-anticoagulant.” A nearly identical profile was observed with normal human plasma (data not shown). Further validation of “NASP” activity was demonstrated by evaluation of three compounds in an APTT clotting assay with Hem A plasma. Concentrations producing approximately 50% prolongation in clotting time were 10– or 100– or >500-fold higher for fucoidan, PPS, and NAH, respectively, than for heparin (Fig. 1B).

**Inhibition of TFPI activity by NASPs**

An assay was established wherein regulation of TFPI activity could be assessed in a physiologically relevant setting. Specifically, a dPT clotting assay with normal or hemophilic plasma and added recombinant TFPI was utilized. TFPI at a final added concentration of approximately 0.5 µg/ml prolonged the clotting time from 40 sec to 100–200 sec depending on the experiment and source of human plasma. If TFPI activity was inhibited by sulfated polysaccharide addition, then a shortening of clotting time should be observed (see also [14]). As shown in Figure 2A with Hem A plasma, addition of fucoidan and PPS significantly accelerated clotting time at concentrations ≥1 nM. In contrast, NAH required concentrations of approximately 100 nM to shorten clotting time and heparin (not shown) only prolonged clotting times. Importantly, at optimal concentrations of PPS or fucoidan, the clotting time was shortened to the “no TFPI”, i.e. vehicle control levels, or slightly below, and the breadth of neutralization of TFPI effect spanned at least a 100-fold range (e.g. 5 to 500 nM). The acceleration of clotting times in the dPT test by the NASPs, presumably via inhibition of added TFPI, was similarly demonstrated with Hem B plasma (Fig. 2B) and normal human plasma (data not shown). The rank order of potency between sulfated polysaccharides was identical to the studies with Hem A plasma and the concentration-response profile was nearly identical.

The experiments represented in Figures 2A and 2B involved a brief preincubation of sulfated polysaccharide with TFPI prior to exposure to plasma. To extend the stringency of the test, studies were performed wherein the TFPI was first added to the plasma, followed by sulfated polysaccharide addition. As depicted in Figure 2C with Hem A plasma, the NASPs clearly demonstrated the same property of clotting time acceleration with nearly identical dose-response profiles as in the preincubation studies (Fig. 2A). Interestingly, fucoidan was most potent and the concentration window for significant clotting acceleration was greater than 100-fold. These studies therefore established that certain NASPs such as PPS and fucoidan could exhibit TFPI neutralizing activity, and that such efficacy was demonstrated across a very broad range of concentrations wherein net anticoagulation was not observed.

**Improvement of hemophilic plasma coagulation by NASPs in the absence of TFPI supplementation**

Building on the foundation described above, an ex vivo correlate for therapeutic potential was to assess whether NASP addition to factor-deficient plasma would accelerate the clotting time in a dPT assay in the absence of TFPI supplementation. A procoagulant response, if observed, may be related to neutralization of endogenous TFPI activity (14) which is present in human plasma at approximately 100 ng/ml but which is largely lipoprotein or pla-
weak TFPI-neutralizing activity (Fig. 2A-C) and did not accelerate hemophilic plasma clotting times in the absence of TFPI addition (data not shown). Moreover, three NASPs which failed to show inherent anticoagulant activity at concentrations up to 5000 nM (Fig. 1A; De-N-S-AH, De-N-SH, and NA-De-O-SH) did not exhibit any TFPI-neutralizing activity and likewise failed to accelerate clotting times in the Hem A plasma dPT assay described in Figure 3 (data not shown). Finally, evaluation over

Figure 2: NASP inhibition of TFPI activity in dilute prothrombin time clotting tests. Dose-response relationships for N-acetyl heparin (NAH), fucoidan, and pentosan polysulfate (PPS) in dPT assays with human plasma. Pure, recombinant TFPI was added to the plasma to prolong the clotting time. NASP inhibition of TFPI activity resulted in reduced plasma clotting times (i.e. prevented TFPI-induced anticoagulation). Clotting time of vehicle control in absence of TFPI ranged 44–48 sec and with added TFPI was 151 sec (A), 101 sec (B), and 183 sec (C). In (A) and (B), NASP was briefly preincubated with TFPI prior to addition to plasma. In (C), TFPI was first mixed into plasma and NASP was subsequently added. Hemophilia A plasma was utilized in (A) and (C), whereas hemophilia B plasma used in (B). Results are representative of duplicate determinations from two studies of each format. Data points are mean ± SD. * p ≤ 0.05 by Student’s t test.

The apparent procoagulant activity by NASP, as assessed in the dPT assay described in Figure 3, was extended to other human bleeding disorders by testing Hem B plasma. Similar results to those shown for Hem A in Figure 3 were observed (data not shown). The approach was likewise evaluated for regulation of plasma clotting in Factor VII deficiency. As expected, FVII-deficient plasma failed to clot within 300 sec in a dPT test without FVIIa reconstitution. In a FVIIa titration trial with FVII-deficient plasma, addition of FVIIa to approximately 0.1 nM restored the clotting time to ~170 sec (see also Fig. 4). This FVII variation of the dPT assay mimics some forms of human factor VII-deficiency. As shown in Figure 4, titration of fucoidan and PPS accelerated clotting times and, as observed with Hem A plasma, fucoidan was significantly more potent and effective than PPS. Once again, the therapeutic window was broad: with fucoidan, substantial acceleration of clotting was observed with concentrations ranging 10 nM to 500 nM.

NASPs lacking procoagulant activity

As established in the aforementioned studies, fucoidan and PPS exhibited NASP activity and accelerated coagulation in dPT assays with hemophilic human plasmas with or without TFPI supplementation. It is noteworthy that such behavior is not apparent with all tested NASPs. For example, NAH exhibited only
primary approaches
Clotting regulation in TFPI-deficient plasma
While human plasma TFPI is truncated and lipoprotein-associated and not fully representative of TFPI fractions in the body (9–11), studies were performed with TFPI-depleted plasma.

Mechanistic approaches
Clotting regulation in TFPI-depleted plasma
While human plasma TFPI is truncated and lipoprotein-associated and not fully representative of TFPI fractions in the body (9–11), studies were performed with TFPI-depleted plasma.

Figure 3: NASP acceleration of hemophilia A plasma clotting times. Dilute prothrombin time coagulation assays of unmodified (i.e., no TFPI supplementation) hemophilia A plasma. Evaluation of dose-response for procoagulant effect of positive control (factor VIIa), fucoidan, or PPS. Vehicle control clotting time = 69 sec. Results are representative of duplicate determinations from two studies of each format. Data points are mean ± SD. *p ≤ 0.05 by Student’s t test.

Figure 4: Fucoidan or PPS shorten the clotting time of factor VII-deficient plasma in the dPT assay. Factor VII-deficient human plasma, which does not clot within 300 sec in a dPT assay, was supplemented with 0.1 nM FVIIa to establish a baseline clotting time of 173 (± 2) sec for the saline vehicle control. Clotting time was measured following preincubation with varying final concentrations of fucoidan or PPS. Results represent duplicate determinations at each condition. Data points are mean ± SD. *p ≤ 0.05 by Student’s t test.

Fucoidan acceleration of clotting times; dilute PT vs APTT
As select NASPs such as fucoidan were shown to inhibit TFPI activity and yet clotting acceleration could also be observed in TFPI-depleted plasma, it became important to try and localize the procoagulant activity within the clotting cascade. One approach undertaken was to compare fucoidan activity in PT vs APTT assays made more sensitive to regulation by dilution of thromboplastin or APTT reagent, respectively. As depicted in Table 1, fucoidan titration into human plasma yielded the reproducible acceleration of clotting time in the dPT assay but did not affect the dAPTT. Hence, these initial mechanistic studies indicate extrinsic pathway (i.e., VIIa/TF) regulation – either TFPI-dependent or potentially TFPI-independent.

Figure 5: Fucoidan effectively neutralized the prolongation of clotting time by truncated TFPI in the dilute PT assay. While the fucoidan IC50 was approximately 6 nM which is approximately 3-fold higher than that observed with full-length TFPI (Fig. 2 and data not shown), the total TFPI protein concentration was much higher given carboxy-truncated TFPI’s reduced potency in such clotting assays (29).

NASP activity against K1K2 TFPI
In conceiving the use of select NASPs for utility in bleeding disorders, initial expectations were that the negatively-charged NASP might interact most strongly and effectively with the positively-charged TFPI carboxy terminus. A useful tool in probing structural determinants was pure recombinant TFPI 1–160, which contains the Kunitz domains responsible for VIIa/TF and Xa interactions but lacks K3 and the carboxy terminus. As shown in Figure 5, fucoidan effectively neutralized the prolongation of clotting time by truncated TFPI in the dilute PT assay. While the fucoidan IC50 was approximately 6 nM which is approximately 3-fold higher than that observed with full-length TFPI (Fig. 2 and data not shown), the total TFPI protein concentration was much higher given carboxy-truncated TFPI’s reduced potency in such clotting assays (29).

Improved hemostasis of NASP-treated mice
Preliminary studies in Hem A or Hem B mice were undertaken with PPS and fucoidan to assess potential improvement of hemostasis in vivo. As quantitation of administered sulfated polysaccharide plasma concentrations is very difficult and pharmacokinetic data for PPS or fucoidan was not identified in the literature, dosing regimen development was largely empirical. We chose to inject the NASP subcutaneously as frequent dosing is reasonably well tolerated in hemophilic mice and bioavailability from this route for various sulfated polysaccharides has been previously established (34, 35). A twice daily dosing regimen was adopted as PPS and fucoidan half-lifes may be as short as 1–2 hr (34, 35) and initial studies suggested that dosing for several days was preferred over 1–2 days. Several potential endpoints were explored for assessing coagulation regulation in the treated mice including plasma iso-

as perhaps the only feasible, direct means of assessing potential mechanisms for dPT clotting acceleration by NASPs such as fucoidan. The standard dPT assay with or without fucoidan titrations (100, 20, 4, 0 nM) and pooled human plasma or plasma depleted of TFPI. The outcome was of nearly identical magnitudes of accelerated coagulation with either plasma source (23% acceleration at 100 nM for both, 20% for regular plasma vs 15% for TFPI-depleted plasma at 20 nM, and 9% acceleration for both plasmas at 4 nM fucoidan). Hence, improved coagulation in a dPT assay by NASP (at least fucoidan) addition ex vivo would not appear to be dependent upon neutralization of plasma TFPI.
lation for dPT assay, blood sampling for whole blood clotting times (WBCT), acute bleeding times, or longer-term survival following tail snip or transverse incision (33). Of all the endpoints, the only one which was both reproducible and showed changes consistent with coagulation factor status (e.g. normal vs factor-deficient vs factor reconstitution) was survival status following tail cut (see also [33]). Survival did not change after the late (20 hr) time point in reported studies.

The results from 5-day in vivo studies with PPS and fucoidan are summarized in Tables 1 and 2. Treatment of Hem A mice with PPS at 0.02, 0.06, and 0.2 mg/kg showed an improvement in survival which was nearly double at the intermediate dose but was not statistically significant (0.05<p<0.1) (Table 2). Therapeutic benefit was further supported by visual observations by technical staff blinded to treatment group who observed more normal behavior (less lethargy and hunching) and less extensive bleeding in the mid and high dose animals relative to the vehicle controls. Likewise, subsequent treatment of Hem B mice with the more effective dose of 0.06 mg/kg subcutaneously twice daily yielded an identical result as that observed in the FVIII-deficient mice.

Given the improved potency and magnitude of efficacy of fucoidan relative to PPS in some of the clotting assays described above, additional studies were performed in Hem A mice with fucoidan (Table 3). In the first study adopting nearly the same regimen as described for PPS, but with slightly different dose levels, we observed improved survival and animal behavior relative to vehicle controls at both 0.1 and 1.0 mg/kg fucoidan dose levels with 0.1 mg/kg trending more efficacious (survival at ~10 hr = 1/6 for vehicle, 4/6 for 0.1 mg/kg, and 3/6 for 1.0 mg/kg). Hence, another study was performed with the vehicle and 0.1 mg/kg dose levels as well as two other groups described below. As indicated in the top two rows of Table 3 which capture these studies, fucoidan treatment of Hem A mice significantly improved bleeding survival. Animal behavior, as described above, was more normal in all the fucoidan-treated mice during the first 8–10 hr post-incision and was clearly improved long-term in nearly half the animals.

Combination therapy potential was assessed by treating mice with FVIII +/- fucoidan (Table 3). A preliminary dose-guiding study with FVIII administration alone to Hem A mice two hours prior to tail incision indicated a very steep dose-response relationship for survival. ReFacto® administration to 1% of normal yielded ~10% survival whereas dosing to 2% of normal yielded ~100% survival (data not shown). Accordingly, a dose of 1.25% FVIII reconstitution was selected to give approximately 50% survival. Notably, the percent survival in the fucoidan + FVIII treatment group was consistently higher than either fucoidan or FVIII alone. Thus, results of the PPS and fucoidan studies support the concept of improved hemostasis in animals models of hemophilia following select NASP administration.

**Discussion**

A series of studies were undertaken to test a well-known class of molecules for a novel biological property: the improvement of clotting in ex vivo and in vivo hemophilia models. Sulfated polysaccharides were identified with substantially reduced anti-

![Figure 5: Fucoidan inhibition of K1K2-TFPI (1–160) activity. Dilute prothrombin time coagulation assay with normal human plasma and pure, recombinant K1K2-TFPI (AA 1–160) with 1:300 simplastin and K1K2-TFPI at 40–50 ug/ml. Vehicle control clotting time 60 sec. Results are representative of duplicate determinations. Data points are mean ± SD. *p≤ 0.05 by Students t test.](https://www.thrombosis-online.com/image)

**Table 1: Fucoidan acceleration of clotting times; dilute PT vs APTT. Clotting assays with normal human plasma.**

<table>
<thead>
<tr>
<th>Concentration (nM)</th>
<th>Dilute PT</th>
<th>Dilute APTT</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.1</td>
<td>3</td>
<td>0</td>
</tr>
<tr>
<td>0.8</td>
<td>6</td>
<td>2</td>
</tr>
<tr>
<td>4</td>
<td>9&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1</td>
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<tr>
<td>20</td>
<td>21&lt;sup&gt;a&lt;/sup&gt;</td>
<td>3</td>
</tr>
</tbody>
</table>

<sup>a</sup> mean of duplicate values for: (vehicle control – fucoidan addition/vehicle control)100; <sup>b</sup>Dilute prothrombin time clotting assay with 1:300 simplastin; <sup>c</sup>Dilute activated partial thromboplastin time with 1:300 APTT reagent.

**Table 2: Improved hemostasis in PPS-treated hemophilic mice.** Mice were randomized and dosed subcutaneously with indicated agent twice daily for 4.5 days followed by tail cut (t = 0).

<table>
<thead>
<tr>
<th>Hemophilia</th>
<th>Treatment Group</th>
<th>n/group</th>
<th>% Survival (20 hr post-cut)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A (FVIII-deficient)</td>
<td>Vehicle control</td>
<td>8</td>
<td>25</td>
</tr>
<tr>
<td></td>
<td>0.02 mg/kg</td>
<td>5</td>
<td>44&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>0.06 mg/kg</td>
<td>9</td>
<td>44&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>0.2 mg/kg</td>
<td>5</td>
<td>40</td>
</tr>
<tr>
<td>B (FIX-deficient)</td>
<td>Vehicle control</td>
<td>8</td>
<td>25</td>
</tr>
<tr>
<td></td>
<td>0.06 mg/kg</td>
<td>9</td>
<td>44&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

<sup>a</sup> p = 0.07 vs vehicle

**Table 3: Efficacy of fucoidan and combination fucoidan + FVIII in hemophilia A mice.** Mice were randomized and dosed subcutaneously with vehicle or NASP twice daily for 4.5 days followed by tail vein incision (t = 0). Where indicated, FVIII was administered 2 hr prior to tail cut. Note that 1% FVIII reconstitution yields ~10% survival whereas 2% FVIII reconstitution provides ~100% survival in these mice.

<table>
<thead>
<tr>
<th>Treatment Group</th>
<th>n/group</th>
<th>% Survival (20 hr post-cut)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vehicle control</td>
<td>14</td>
<td>7</td>
</tr>
<tr>
<td>Fucoidan</td>
<td>13</td>
<td>61&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Factor-VIII</td>
<td>13</td>
<td>57&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Fucoidan + FVIII</td>
<td>7</td>
<td>86&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
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</table>

<sup>a</sup> p < 0.05 vs vehicle; <sup>b</sup> p = 0.06 vs vehicle; <sup>c</sup> p = 0.06 vs fucoidan
coagulant properties relative to heparin. A subset of those NASPs, namely fucoidan and PPS, were shown to potently inhibit the activity of TFPI, the predominant downregulator of the extrinsic pathway of blood coagulation. Fucoidan and PPS improved the dilute prothrombin clotting times of human plasma deficient in factors VII, VIII, or IX. Therapeutic benefit of fucoidan or PPS treatment in vivo was apparent from bleeding tests of hemophilic mice.

Sulfated polysaccharides are a structurally diverse class and have many reported activities. Heparinoid polysaccharides, which include pentosan polysulfate, are derived from animal, plant, or synthetic sources and are generally dependent upon heparin-binding protein interactions for biological activity (17). PPS has been used in Europe as an anticoagulant (36), was tested for anti-tumor activity in the clinic (37), and is clinically effective for interstitial cystitis – the indication for which it is approved and marketed (31, 38). Fucoidan is polymeric sulfated L-fucose which is often isolated from plant tissue and has been associated with anti-inflammatory, anti-proliferative, and antiviral activities (17, 39). While both PPS and fucoidan may exhibit anticoagulant activity, likely as a result of heparin cofactor II interaction (40, 41), PPS administered subcutaneously to rats requires doses >5 mg/kg to prolong clotting (41) and fucoidan seems well-tolerated in rabbits even when given intravenously at 10 mg/kg (42). Hence, the current results supporting improved hemostasis at doses ≤0.1 mg/kg in hemophilic rodents represent a novel biological property for certain sulfated polysaccharides which occurs at dose levels in vivo lower than other reported effects (17, 26, 39, 42, 43).

Limited conclusions can be derived from the current set of data regarding sulfated polysaccharide structure-activity relationships. Results demonstrating inactivity of acetylated and desulfated heparins along with efficacy of fucoidan and PPS suggest that the second position of the amino sugar may be important for TFPI inhibition and/or coagulation acceleration of hemophilic plasma. As compound evaluation was not extensive, there may well be more effective compounds or modifications of active molecules which should be tested including apparent NASPs described in the literature such as GM1474 or low molecular weight fucoidan (22, 44).

In terms of the initially-conceived mechanism relating NASP inhibition of TFPI activity as the means for supporting improved extrinsic pathway activation potential, direct approaches for testing the hypothesis yielded mixed results. In support of the relationship, neutralization of TFPI by antibodies has been shown to improve hemostasis in a rabbit Hem A model and to accelerate clotting of human hemophilic plasma (14–16). In the current studies, only compounds inhibiting TFPI activity also reduced clotting times in the hemophilic plasma dPT assays and fucoidan, specifically, exhibited neutralizing activity against full-length and truncated TFPI. Additionally, fucoidan exhibited better potency and perhaps greater maximal effect compared to PPS in the dPT clotting test when the TFPI was first mixed into plasma to best mimic the natural setting. Likewise, fucoidan treatment of mice yielded somewhat better efficacy than PPS, although undefined relative pharmacokinetics may have influenced the bleeding outcomes. Data opposing a link between NASP procoagulant activity and TFPI inhibition derived from the activity of fucoidan in TFPI-depleted plasma. Full-length TFPI, which is more endothelium-localized and the key component in extrinsic pathway (and bleeding) regulation in vivo (9–11), might still be an important component of NASP (fucoidan) improvement of hemophilic subject hemostasis. Nonetheless, the ex vivo dPT clot acceleration by fucoidan and PPS must possess an element of TFPI-independent action.

If TFPI inhibition were only a component of NASP-improved clotting ex vivo and in vivo, how else might coagulation be affected consistent with the profiles described? Efficacy might involve numerous regulatory actions including Xa or thrombin generation/activity, platelet aggregation, and/or fibrinolysis. In fact, sulfated polysaccharides including pentosan polysulfate have been shown to influence fibrinogen binding to platelets (45) and the release of plasminogen activator (46). Moreover, the high (150 kDa) molecular weight fraction of fucoidan has been shown to enhance plaquelet activation in vitro (47).

One ex vivo approach, to help localize potential coagulation cascade regulation, involved comparison of fucoidan activity in dPT vs dAPTT assays. The clear, concentration-dependent acceleration of clotting in the dPT without any notable change in the dAPTT would imply more upstream and perhaps more extrinsic pathway-related action. Accordingly, one potentially unifying model may be that select NASPs such as fucoidan alter activity of the FVIIa/TF complex (including co-factors) such that the outcome is increased extrinsic pathway activation and less apparent TFPI activity. Further dissection of actual mechanisms in vivo and ex vivo for hemostasis regulation by NASPs such as fucoidan and PPS will require additional testing in multiple model systems.

In considering this novel approach for treating hemophilia, one might question whether the magnitude of improved hemostasis observed, let alone achievable, is clinically relevant. As described in Results pertaining to Figure 3, improved clotting times of Hem A plasma at optimal fucoidan concentrations were comparable to FVIIa supplementation at approximately 5 nM which has proven effective in normalizing hemostasis in patients (1–4, 48). In terms of observed efficacy in vivo, it would be desirable to have shown 100% survival in the hemophilic mice with NASP treatment alone. Nonetheless, under the conditions tested, the improved bleeding survival by fucoidan was substantial in that it was comparable to supplementation with FVIII to 1.25% of normal (Table 2) which is notable because reconstitution to 2% of normal FVIII in Hem A mice reproducibly yielded 100% bleeding survival. Survival benefit in hemophilic mice by fucoidan treatment was therefore significant, and it is feasible that greater efficacy could be achieved with further optimization of the dosing regimen, let alone the test compound itself. Importantly, it is likely that the magnitude of improved hemostasis in humans by select NASP(s) may be greater than that in mice: first, fucoidan acceleration of clotting in dPT assays appears more pronounced with human hemophilic plasma than mice (data not shown); second, TFPI is speculated to be present at higher concentration in mice vs humans (J. Wun, G. Broze unpublished data) and, therefore, the threshold to overcome may be higher in mice. Finally, one must consider that even if the improvement in coagulation in humans were of a modest magnitude, such a response might be
acceptable as too strong of a procoagulant effect might yield undesirable pro-thrombotic episodes.

An obvious consideration regarding potential clinical development of a NASP for bleeding disorders would be therapeutic index. Specifically, index between improved hemostasis and the transition to anti-coagulation. From the clotting assay results for compounds such as PPS or fucoidan, the margin between anti-TFPI or accelerated dPT clotting “activity” and loss of such efficacy and onset of net anticoagulation would appear to be ≥50-fold. As mentioned above for the mouse studies, the index would appear to be at least ten-fold. Furthermore, as a class, heparin-like sulfated polysaccharides are generally well tolerated. Potential side-effects of a NASP treatment could prove both adverse or beneficial. An undesirable side-effect might include the well-known complication of heparin-induced thrombocytopenia. However, such low concentrations of non-heparin structures – i.e., fucoidan – may not be similarly inclined. While an additional adverse effect could conceivably include thrombosis, such an outcome might not be anticipated given the level of correction being observed so far and really can not be fairly assessed until both dosing regime and specific NASP are optimized. Alternatively, favorable side-effects might be observed in hemophilia patients as heparin and related molecules have shown beneficial anti-inflammatory or cardiovascular properties (17, 19, 21, 34, 43, 44).

In summary, a novel approach for regulating clotting in bleeding disorders has been described which involves non-anticoagulant sulfated polysaccharides. Support for improved hemostasis was provided by prototypes including pentosan polysulfate and fucoidan. With further chemical and pharmacological development efforts, these compounds or others may become relatively low-cost, safe, and convenient alternatives or supplements to factor therapies.

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