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BIOCHEMICAL INVESTIGATION OF THE ANTICOAGULANT ACTIVITY OF SULFONATED POLYMERS

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Abstract

Venous thrombotic disorders are a leading cause of mortality in the US. While anticoagulants are the primary treatment, their use is limited by bleeding risks. This highlights the critical need for safer alternatives. In this study, we investigated eight sulfonated polymers for anticoagulant potential using human plasma clotting assays. Nonacetylated lignosulfonate (NALS) emerged as the most promising candidate, doubling clotting times at 309–980 µg/mL concentrations. We also performed chromogenic assays targeting key coagulation factors (thrombin, factor Xa, and factor XIa) to elucidate its mechanism of action. NALS exhibited a potent and selective inhibition of factor XIa, with an IC_{50} of ~8 µg/mL, while showing minimal effects on thrombin or factor Xa. Factor XIa inhibition is particularly advantageous, as it may reduce thrombosis while preserving hemostasis, addressing a major drawback of current therapies. Our findings position NALS as a novel, targeted anticoagulant lead with a mechanistically improved safety profile. Future studies will explore its efficacy in vivo and structure-activity relationships to optimize therapeutic potential. This work opens new avenues for developing safer antithrombotic drugs.

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Introduction

Thromboembolic diseases encompass arterial thrombosis (AT) and venous thrombosis (VT). AT comprises conditions such as ischemic heart disease and stroke, with ischemic heart disease being the leading cause of cardiovascular-related deaths in the U.S. (45.1%), followed by stroke (16.5%)[1]. In contrast, VT includes deep vein thrombosis and pulmonary embolism, accounting for approximately 274 daily deaths in the U.S. VT is the third most common vascular diagnosis linked to cardiovascular mortality, contributing to over 3.2% of such deaths[2,3]. VT annually affects around 1 million individuals, a figure projected to rise to 1.82 million by 2050[4]. Furthermore, cancer patients face a 4–7 times higher risk of developing VT, representing 15–20% of all VT cases[5]. Interestingly, African Americans in the U.S. have a higher incidence of VT compared to other racial and ethnic groups[6].

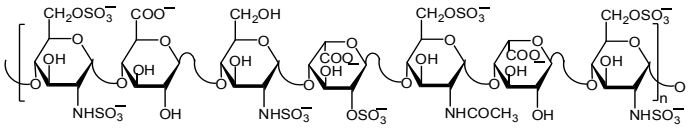
Therapeutic approaches differ for AT and VT. Antiplatelet agents are used for AT, while anticoagulants are employed for VT (Figure 1). The coagulation cascade involves a sequence of enzymatic reactions primarily driven by serine proteases, including factors IIa, VIIa, IXa, Xa, XIa, and XIIa (with factor XIIIa being a transglutaminase). Coagulation can be initiated via either the intrinsic or the extrinsic pathway (Figure 2). Current anticoagulants target thrombin and/or factor Xa, which explains their efficacy but also their major drawback of the increased bleeding risk. In contrast, factors XIa and XIIa play critical roles in thrombosis but not in hemostasis, making them promising targets for safer anticoagulation, as their inhibition reduces thrombosis without impairing normal clotting[7-10].

In this study, we investigated the anticoagulant potential of various sulfonated polymers by assessing their effects on human plasma clotting times (activated partial thromboplastin time, APTT, and prothrombin time, PT). Our goal was to identify a novel non-saccharide chemical scaffold for further development as an anticoagulant. Considering availability, the most promising candidate was then evaluated for its inhibitory effects on thrombin, factor Xa, and factor XIa using chromogenic substrate assays. Among the tested compounds, nonacetylated lignosulfonate (NALS) emerged as a standout, doubling clotting times in human plasma at concentrations of ~310 µg/mL (APTT) and ~980 µg/mL (PT). Furthermore, NALS demonstrated selective inhibition of factor XIa with an IC_{50} of 8 µg/mL, highlighting its potential as a lead compound for further development of a safer anticoagulant therapy.

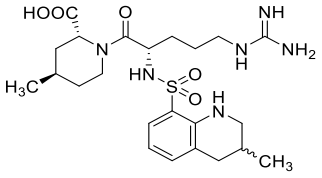
Experimental Methods

Materials

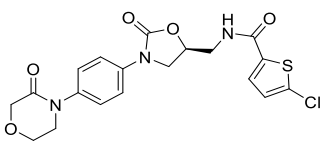
Unfractionated heparin (UFH; Mwt ~15,000), nonacetylated



Representative structure of UF and LMWHs;
(Antithrombin activators)



Argatroban
(Active site thrombin inhibitor)



Rivaroxaban
(Active site FXa inhibitor)

Figure 1. The chemical structures of currently available anticoagulants, along with their mechanism of action.

lignosulfonate (NALS; Mwt ~52,000), acetylated lignosulfonate (ALS), poly(4-styrenesulfonate) (PSS; Mwt ~70,000 and 200,000), poly(4-styrenesulfonate-co-maleic acid) (PSSM; Mwt ~20,000), polyanethol sulfonate (PAS), and polyvinylsulfonate salts (PVS, potassium and sodium salts) were obtained from Sigma-Aldrich (St. Louis, MO). Human plasma clotting enzymes for inhibition studies were purchased from Haematologic Technologies (Essex Junction, VT), while chromogenic substrates for thrombin and factor Xa were acquired from Biomedica Diagnostics (Windsor, NS, Canada), and the factor XIa substrate (S-2366) was sourced from Diapharma (West Chester, OH). Stock solutions of thrombin and factor XIa were prepared in 50 mM Tris-HCl buffer (pH 7.4) containing 0.02% Tween 80, 0.1% PEG 8000, and 150 mM NaCl, whereas factor Xa was prepared in 20 mM Tris-HCl buffer (pH 7.4) with 100 mM NaCl, 2.5 mM $CaCl_2$, 0.1% PEG 8000, and 0.02% Tween 80. Human plasma for clotting time assays (APTT and PT) was obtained from George King Bio-Medica (Overland Park, KS), and APTT reagent (containing ellagic acid), PT reagent (thromboplastin-D), and 0.025 M $CaCl_2$ solution were purchased from Thermo Fisher Scientific (Waltham, MA, USA). All experiments were conducted in triplicate.

Effect of Sulfonated Polymers on Clotting Times of Human Plasmas

The anticoagulant activity of enzyme inhibitors is commonly evaluated using plasma clotting assays, specifically the activated partial thromboplastin time (APTT) and prothrombin time (PT). APTT assesses the intrinsic pathway (involving factors XIIa, XIa, and IXa), while PT evaluates the extrinsic pathway (mediated by factor VIIa). These experiments were performed using a BBL Fibrosystem fibrometer (Becton-Dickinson, Sparks, MD) following established protocols[11-13]. For the APTT assay, 90 µL of human plasma was combined with 10 µL of a sulfonated polymer (or vehicle control) and 100 µL of prewarmed 0.2% ellagic acid. After incubating for 4 minutes at 37°C, clotting was initiated by adding 100 µL of prewarmed 0.025 M $CaCl_2$, and the clotting time was recorded. In the PT assay, thromboplastin-D reagent was prepared by reconstituting with 4 mL of distilled water and prewarming to 37°C. A 90 µL aliquot of plasma was mixed with 10 µL of the test polymer (or vehicle), incubated for 30 seconds at 37°C, and then 200 µL of prewarmed thromboplastin-D was added to trigger clotting. Both assays employed at least nine polymer concentrations to generate concentration-response curves, with data fitted to a quadratic trendline to determine the concentration required to double the clotting time. Negative controls using 10 µL of purified water were run in parallel.

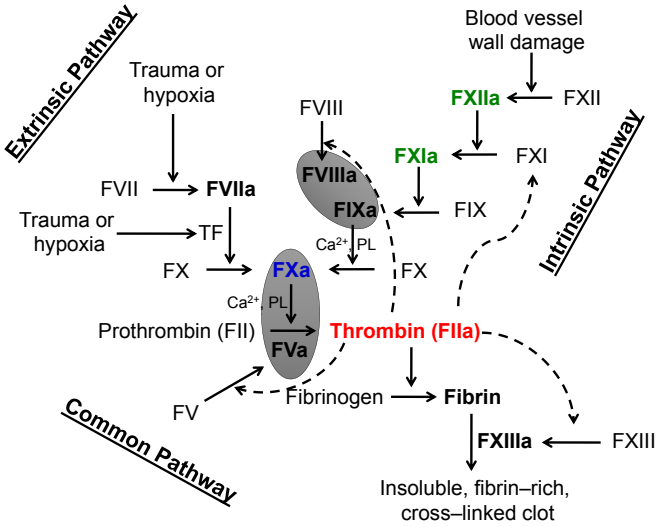


Figure 2. The waterfall model of the coagulation process.

Additional experiments were conducted using antithrombin-deficient human plasmas to investigate the role of antithrombin (the target of unfractionated heparin, UFH) in the APTT assay. Positive controls included: (1) UFH (an antithrombin activator), (2) argatroban (a clinical thrombin inhibitor), (3) rivaroxaban (a clinical factor Xa inhibitor), and (4) anti-F11 (a factor XIa-inhibiting antibody).

Effect of NALS on Thrombin, Factor Xa, and Factor XIa

The inhibitory effects of NALS on thrombin, factor Xa, and factor XIa were evaluated using established chromogenic substrate hydrolysis assays, as reported in previous experiments[14-16]. In these experiments, 5 µL of NALS (0-5.0 mg/mL) or vehicle control was combined with 5 µL of enzyme in a 96-well microplate containing 85-185 µL of Tris-HCl buffer (20-50 mM, pH 7.4) supplemented with 0.02% Tween 80, 0.1% PEG 8000, and 100-150 mM NaCl. Assays were conducted at 37°C for factors Xa and XIa, and at 25°C for thrombin, using final enzyme concentrations of 1.09 nM (factor Xa), 0.765 nM (factor XIa), and 6 nM (thrombin). After 10 minutes of incubation, the appropriate chromogenic substrate was added (125 µM for factor Xa, 345 µM for factor XIa, or 50 µM for thrombin). Residual enzyme activity was quantified by measuring the initial rate of absorbance increase at 405 nm. For cases showing significant inhibition, the relationship between NALS concentration and residual enzyme activity was analyzed using Equation (1).

$$Y = Y_0 + \frac{Y_M - Y_0}{1 + 10^{(log[Inhibitor]_0 - log\ IC_{50}) \times HS}} \qquad (1)$$

In this equation, Y represents the fractional residual enzyme activity (relative to control), Y_0 and Y_M denote the minimum and maximum residual activity values, IC_{50} is the NALS concentration yielding 50% inhibition, and HS is the Hill slope. These parameters were determined through nonlinear regression analysis of the experimental data.

Results and Discussion

We assessed the anticoagulant properties of eight sulfonated polymers (**1**-**8**, Figure 3) using standard plasma clotting assays. The activated partial thromboplastin time (APTT) and prothrombin time (PT) assays were employed to evaluate intrinsic (factors IXa, XIa, XIIa) and extrinsic (factor VIIa) pathway inhibition, respectively. These studies were conducted in both normal human plasma and antithrombin-deficient plasma following established protocols[17]. The tested polymers comprised aromatic polystyrene sulfonates (PSS; polymers **1** and **2**) of varying molecular weights, lignosulfonate derivatives (acetylated ALS, polymer **3**; nonacetylated NALS, polymer **7**), poly(4-styrenesulfonate-co-maleic acid) (PSSM, polymer **4**), polyanethol sulfonate (PAS, polymer **5**), and non-aromatic polyvinylsulfonate sodium and potassium salts (PVS, polymers **6** and **8**).

As shown in Table 1, the polymers exhibited distinct anti-

coagulant profiles. The most potent compounds were polymer **2** (APTT-doubling concentration: 1.94 µg/mL; PT: 4.22 µg/mL) and polymer **4** (APTT: 1.31 µg/mL; PT: 4.26 µg/mL). However, we focused on NALS (polymer **7**) due to its industrial availability and cost-effectiveness as an abundant byproduct. NALS demonstrated concentration-dependent anticoagulant activity, doubling APTT at 309 ± 24 µg/mL in normal plasma (Figure 4A) and 275 ± 62 µg/mL in antithrombin-deficient plasma. Notably, PT required significantly higher NALS concentrations (~980 µg/mL), suggesting preferential intrinsic pathway targeting. This selectivity (>3-fold APTT vs PT effect) resembled the profile of factor XIa inhibitors rather than thrombin or factor Xa inhibitors. Comparative studies with clinical anticoagulants revealed that UFH showed antithrombin-dependent activity (APTT: 0.7 µg/mL normal plasma vs 10 µg/mL deficient plasma) (Figure 4B), argatroban (thrombin inhibitor) and rivaroxaban (factor Xa inhibitor) affected both pathways equally, and NALS' profile closely matched anti-F11 (factor XIa inhibitor).

Chromogenic assays confirmed NALS selectively inhibited factor XIa (IC_{50} = 8 ± 1 µg/mL, 98 ± 5% efficacy) (Figure 4C) without affecting thrombin or factor Xa at concentrations up to 125 µg/mL (selectivity index >16). It is important to emphasize here that the clinical anticoagulant effect of UFH primarily arises from its ability to enhance antithrombin activity against various clotting factors. Although UFH can also bind directly to factors like XIa, this interaction is not clinically significant, as reflected by its relatively lower potency (IC_{50} = 3.15 ± 0.75 µg/mL) and modest

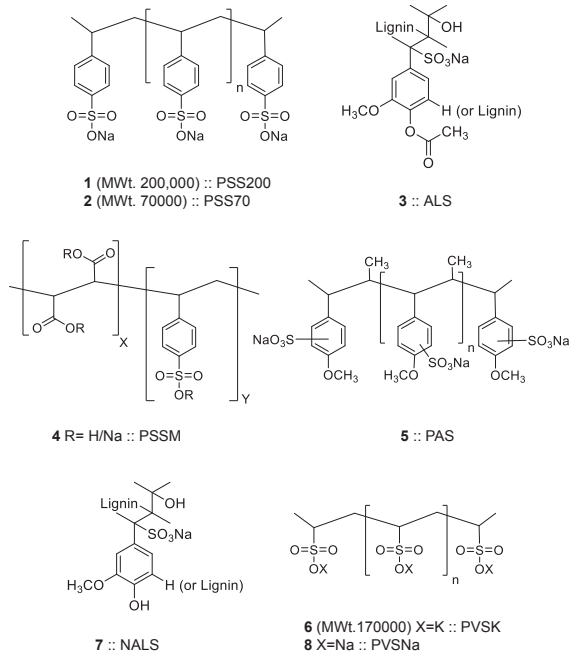


Figure 3. The chemical structures of sulfonated polymers 1-8 used in this study.

Table 1. Effects of sulfonated polymers 1-8 on APTT and PT.

Sulfonated polymer	APTT (EC_{2X}) µg/mL (µM)	PT (EC_{2X}) µg/mL (µM)
UFH	0.68 ± 0.08	2.53 ± 0.2
Argatroban	(0.17 ± 0.06)	(0.18 ± 0.05)
Rivaroxaban	(0.052 ± 0.01)	(0.065 ± 0.01)
Anti-F11	1.99 ± 0.2	>>3.6
1 (PSS200)	3.4 ± 0.4	62.38
2 (PSS70)	1.94 ± 0.3	4.22
3 (ALS)	43.3 ± 5	206.64
4 (PSSM)	1.31 ± 0.07	4.26
5 (PAS)	20.34 ± 2	35.37
6 (PVSK)	>550	>550
7 (NALS)	309 ± 24	980 ± 145
8 (PVSNa)	11.78 ± 1.2	339.3 ± 30

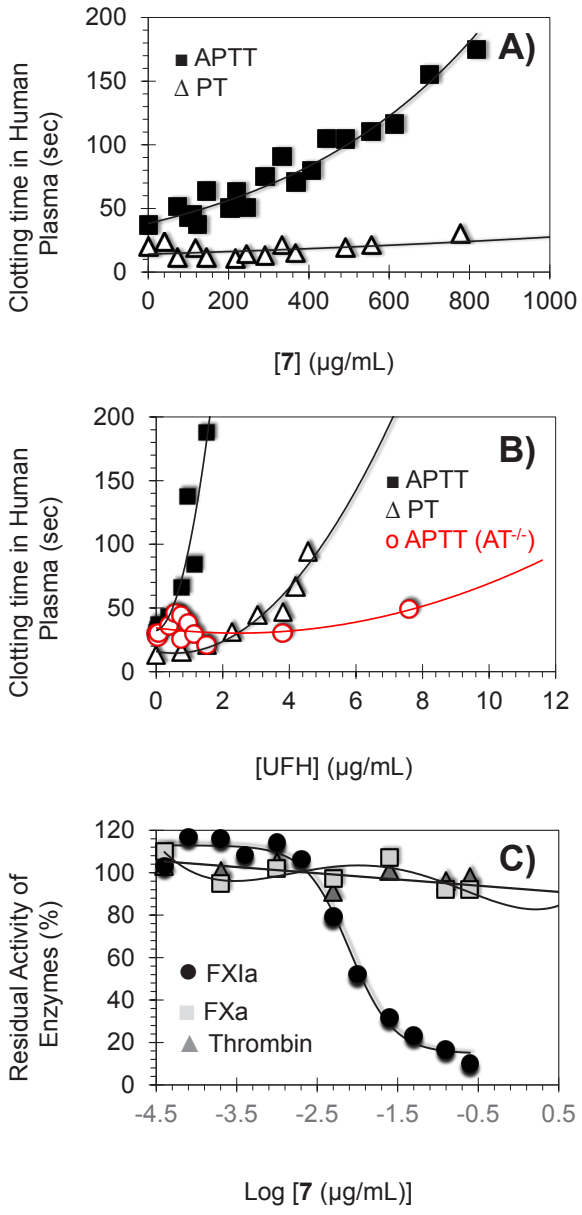


Figure 4. A) The effect of NALS on APTT and PT of normal human plasma. B) The effect of UFH on APTT and PT of normal and antithrombin-deficient human plasmas. C) The inhibition profile of NALS against human factor XIa, thrombin, and factor Xa in chromogenic substrate hydrolysis assays.

efficacy (30%) (Figure 5). Together, these findings underscore the unique and potent anticoagulant potential of NALS, which functions independently of antithrombin by directly and selectively inhibiting factor XIa.

Although it is still too early to draw definitive structure-activity relationships, several trends are emerging. First, the 4-styrenesulfonate-containing polymers **1** and **2** show comparable effects on APTT; however, polymer **1** (with a higher molecular weight) exhibits greater selectivity for the intrinsic pathway, as indicated by a PT/APTT ratio of 18.3. In contrast, polymer **2** (with a lower molecular weight) impacts both the intrinsic and extrinsic pathways nearly equally (PT/APTT ratio = 2.2). To assess the role of the aromatic component, comparing polymer **1** with polymer **8** (which has a similar molecular weight) is informative. Both polymers selectively affect the intrinsic pathway, with PT/APTT ratios of 18.3 and 33.9, respectively. Nevertheless, the aromatic polymer **1** is approximately three times more potent by weight than the non-aromatic polymer **8**. It is also important to note that the sodium salt form of these polymers is the active species. This is evident from the comparison between the inactive potassium salt (polymer **6**) and the active sodium salt (polymer **8**). Additionally, despite structural similarity between polymers **2** and **5**, the inclusion of methyl and methoxy groups in polymer **5** reduces its potency by about 11-fold (by weight) without affecting its selectivity (PT/APTT ratio = 1.7). Interestingly, adding two carboxylate groups to poly(4-styrenesulfonate) (polymer **4**) does not alter its potency or selectivity compared to polymer **2**. Meanwhile, lignin-based polymers **3** and **7** are less potent overall but offer a cost advantage, especially polymer **7**, which is a byproduct of industrial sulfite pulping[18]. Although the acetylation of polymer **7** (NALS) produces the more potent polymer **3** (ALS), the latter lacks enzyme-level selectivity (Table 2). Chromogenic substrate hydrolysis assays revealed that polymer **3** inhibits thrombin, factor Xa, and factor XIa uniformly, whereas polymer **7** shows a preference for inhibiting factor XIa. Overall, the selectivity, the cost advantage, and the chemical scaffold that is amenable to modifications make polymer **7** (NALS) a more promising candidate for the development of safer anticoagulants with a lower risk of bleeding.

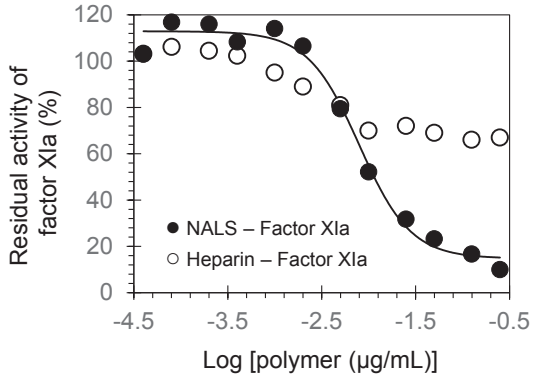


Figure 5. Comparative inhibition of factor XIa by NALS (polymer **7**) and UFH.

Table 2. The selectivity of ALS and NALS toward thrombin, factor Xa, and factor XIa using the corresponding chromogenic substrate hydrolysis assay.

Polymer	Enzyme	Inhibition Parameters
ALS (polymer **3**)	**Thrombin**	IC_{50}=0.73± 0.03 µg/mL HS=1.2 ±0.2 ΔY=80.9 ±1.9%
	Factor Xa	IC_{50}=0.48 0.09 µg/mL HS=0.94 ±0.17 ΔY=87±6%
	Factor XIa	IC_{50}=0.39 ±0.1 µg/mL HS=1.6 ±0.3 ΔY=70.9±7.5%
NALS (polymer **7**)	**Thrombin**	>125 µg/mL
	Factor Xa	>125 µg/mL
	Factor XIa	IC_{50}= 8.0±1.0 µg/mL HS=0.9 ±0.2 ΔY=98.1±4.7%

Conclusions and Future Directions

Our study identified NALS, an industrial lignin byproduct, as a potent and selective polymeric inhibitor of human factor XIa. This enzyme represents an ideal target for next-generation anticoagulant development since it plays a crucial role in thrombosis while being relatively unimportant for normal hemostasis, potentially offering an improved safety profile with reduced bleeding risk compared to current therapies[19,20]. NALS demonstrates remarkable specificity for factor XIa over conventional anticoagulant targets (thrombin and factor Xa), along with several practical advantages that enhance its therapeutic potential. As an abundant industrial byproduct, NALS offers significant economic advantages for large-scale production. Its production avoids the contamination risks associated with heparin manufacturing, particularly the potential presence of supersulfated polysaccharides that have caused supply chain issues. Importantly, NALS shows excellent safety characteristics, including minimal acute toxicity (oral LD_{50} = 6,030 mg/kg in mice)[21] and the inherent safety advantage of targeting factor XIa rather than essential hemostatic factors.

The unique properties of NALS make it particularly promising for treating high-risk patient populations who require anticoagulation but are vulnerable to bleeding complications, such as those with chronic kidney disease or atrial fibrillation[22-26]. Given these advantages, future research will focus on developing optimized small-molecule analogs based on the NALS structure. These studies will explore structure-activity relationships to identify key pharmacophores while maintaining the favorable selectivity profile of the parent compound. The ultimate goal is to develop clinically viable, sulfonated small-molecule factor XIa inhibitors that retain the safety advantages of NALS while offering improved pharmaceutical properties. This approach may lead to a new class of anticoagulants that effectively prevent thrombosis without disrupting normal hemostasis, addressing a critical unmet need in cardiovascular medicine. In addition, we will conduct in vivo studies using multiple thrombosis models, including ferric chloride-induced and stenosis-induced thrombosis, to further assess the safety and efficacy of NALS in physiologically relevant settings. While NALS exhibits sufficient solubility for parenteral administration, oral delivery remains a highly desirable yet challenging route due to the polymer's limited gastrointestinal absorption. To address this, NALS can be structurally modified through conjugation with lipids or bile acids to enhance its lipophilicity and, consequently, its oral bioavailability. Encapsulation in micro- or nanoparticles and the incorporation of absorption enhancers such

as sodium N-(8-[2-hydroxybenzoyl]amino) caprylate (SNAC) are also promising strategies. Furthermore, alternative delivery routes will be explored: pulmonary delivery, via inhalable formulations, offers a non-invasive option for localized lung treatment, while topical delivery using NALS-based formulations may provide targeted therapy for conditions such as superficial thrombosis or wound healing[27,28]. The discovery of NALS as a factor XIa inhibitor therefore represents an important step toward safer anticoagulation therapies, combining practical manufacturing advantages with an innovative mechanism of action.

Acknowledgments

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