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Effect of heparanase inhibitor on tissue factor overexpression in platelets and endothelial cells induced by anti-β2-GPI antibodies

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Abstract

Background: Anti-phospholipid syndrome (APS) is characterized by arterial and/or venous thrombosis and pregnancy morbidity associated with the presence of "anti-phospholipid antibodies." Thrombosis may be the result of a hypercoagulable state related to activation of endothelial cells and platelets by anti-β2-glycoprotein I (β2-GPI) antibodies. Anti-β2-GPI antibodies induce a proinflammatory and procoagulant phenotype in these cells that, after activation, express tissue factor (TF), the major initiator of the clotting cascade, playing a role in thrombotic manifestations. Moreover, TF expression may also be induced by heparanase, an endo-β-D-glucuronidase, that generates heparan sulfate fragments, regulating inflammatory responses.

Objectives: In this study we analyzed, in human platelets and endothelial cells, the effect of a new symmetrical 2-aminophenyl-benzazolyl-5-acetate derivative (RDS3337), able to inhibit heparanase activity, on signal transduction pathways leading to TF expression triggered by anti-β2-GPI.

Methods: Platelets and endothelial cells were incubated with affinity purified anti-β2-GPI after pretreatment with RDS3337. Cell lysates were analyzed for phospho-interleukin-1 receptor-associated kinase 1 (IRAK1), phospho-p65 nuclear factor kappa B (NF-κB) and TF by western blot. In addition, platelet activation and secretion by ATP release dosage were evaluated.

Results: IRAK phosphorylation and consequent NF-κB activation, as well as TF expression triggered by anti-β2-GPI treatment were significantly prevented by previous pretreatment with RDS3337. In the same vein, pretreatment with RDS3337 prevented platelet aggregation and ATP release triggered by anti-β2-GPI antibodies.

Conclusion: These findings support the view of heparanase involvement in a prothrombotic state related to APS syndrome, suggesting a novel target to regulate overexpression of procoagulant protein(s).

1 | INTRODUCTION

Anti-phospholipid antibodies (aPL), which include anti-β2-glycoprotein I (β2-GPI), anticardiolipin antibodies (aCL), and/or lupus anticoagulant (LAC) are serological markers of anti-phospholipid syndrome (APS), a systemic autoimmune disease characterized by clinical features including arterial and/or venous thrombosis, early miscarriages, or fetal deaths.[1-3] aPL represent a heterogeneous family of antibodies, including anti-β2-GPI.[3]

Anti-β2-GPI antibodies may be responsible for thrombosis resulting from a hypercoagulable state related to the activation of endothelial cells and platelets. Indeed, anti-β2-GPI antibodies induce a proinflammatory and procoagulant phenotype in these cells which, after activation, express tissue factor (TF), the main initiator of the coagulation cascade.[4] It is already known that the dysfunction of endothelial cells and platelets can play an active role in the pathogenesis of deep vein thrombosis and therefore of APS. In fact, the loss of the glycocalyx, a thin layer rich in glycosaminoglycans (GAG) on the surface of endothelial cells, is a key feature of endothelial dysfunction and increases the exposure of adhesion molecules, such as selectins, which are involved in platelet binding to endothelial cells.[5] Moreover, it was reported that the anti-β2-GPI/β2-GPI complex binds to the platelet thrombus and amplifies platelet activation. In the same paper the authors showed that inhibition of platelet activation prevents the activation of endothelial cells and the formation of fibrin.[6] Recently, we showed that platelets can express TF on their surface. In particular, it was shown that resting unstimulated platelets express TF and this protein is enhanced or induced following cell activation by a signal transduction pathway that involves interleukin-1 receptor-associated kinase 1 (IRAK) phosphorylation and nuclear factor kappa B (NF-κB) activation.[7] Furthermore, platelets from APS patients showed a significantly increased expression of TF.[7] It supported the view that platelets play an important role in the pathogenesis of APS, by activating a signal transduction pathway leading to the release of different procoagulant mediators, as in nucleated cells.[8,9]

Previous data indicate that TF expression may be also induced by heparanase, which is expressed at high levels in placenta, mast cells, neutrophils, lymphocytes, and platelets. Heparanase is an endo-β-D-glucuronidase capable of cleaving heparan sulfate (HS) side chains, both in extracellular space and within the cells, regulating several biological activities. Indeed, in addition to its well-characterized role in cancer, heparanase activity may play a role in the pathogenesis of several inflammatory disorders, promoting migration of vascular endothelial cells and activation of immune system cells.[10,11] Heparanase generates soluble HS fragments that control inflammatory responses at multiple levels, including the release of cytokines/chemokines in the extracellular space, modulation of leukocyte interactions with endothelial cells and extracellular matrix, and initiation of innate immune responses through interactions with Toll-like receptor 4 (TLR-4).[12-14] This mechanism was demonstrated by the significant inhibition of cytokine release using inhibitors of heparanase enzymatic activity. It revealed that heparanase-induced cytokine release was dependent on MyD88, and thus mediated through TLR-4 via the NF-κB pathway.[15,16]

Thereafter, heparanase, besides being recognized as a proinflammatory protein, is involved in wound healing and in activation of the coagulation cascade. It may act as a cofactor of TF and not only upregulates TF expression in endothelial cells, but also directly interacts with cell surface TF and enhances factor Xa production with subsequent activation of the coagulation system.[17] The hemostatic function of heparanase suggests a potential clinical relevance of this protein; in fact, targeting the procoagulant domain of heparanase may be useful for patients with pro-angiogenic and prothrombotic conditions.[18]

Over the years, several inhibitors have been discovered searching molecules able to interfere with the biological activity of heparanase; among these, it is possible to find inhibitors of synthetic and natural origin, several heparin derivatives and polysulfated oligosaccharides, proteins, monoclonal antibodies, and nucleic acids.[19]

The development of some inhibitors of heparanase may exceed some of the limitations of polysaccharides. Due to their properties, to be properly designed to have favorable pharmacokinetic and oral availability, small molecules are particularly desirable. Some benzoxazole derivates, in the group of synthetic small molecules, have been described as heparanase inhibitors; among these benzoxazol-5-yl acetic acid showed promising properties as heparanase inhibitors.[20,21]

Essentials

- In antiphospholipid syndrome anti-β2-glycoprotein I (β2-GPI) antibodies induce a signal transduction pathway that leads to tissue factor (TF) expression on the cell surface.
- We analyze the effect of a new heparanase inhibitor on signal transduction pathways leading to TF expression triggered by anti-β2-GPI in platelets and endothelial cells.
- Signal transduction pathways leading to TF expression as well as platelet aggregation induced by anti-β2-GPI are shown to be prevented by heparanase inhibitor RDS3337.
- These findings suggest a "new" potential therapeutic target to regulate overexpression of procoagulant protein(s) in antiphospholipid syndrome.

In the present study we describe the biological effect of a new symmetrical 2-aminophenyl-benzazolyl-5-acetate derivative able to inhibit heparanase enzymatic activity.

In particular, we investigate the inhibitory role of this new compound on endothelial cells and platelets activation triggered by anti-β2-GPI antibodies. Because heparanase plays a role in thrombosis and endothelial dysfunction, but also through the enzymatic cleavage of HS, we analyze this specific heparanase inhibitor as a molecule affecting the increase of TF induced by anti-β2-GPI antibodies. Thus, we may suggest a novel target to regulate overexpression of procoagulant protein(s) (i.e., TF) and, consequently, the prothrombotic state commonly associated with the pathogenesis of thrombosis during APS.

2 | METHODS

2.1 | Analysis of the effect of heparanase inhibitors on endothelial cell viability

Human umbilical vein endothelial cells (HUVECs) were grown in PromoCell growth medium containing endothelial cell growth medium kit (PromoCell) and 10% fetal bovine serum (Sigma-Aldrich), at 37°C in a humified 5% CO_2 atmosphere.

The benzazolyl derivatives endowed with potent anti-heparanase activity have been designed and developed as previously reported by some authors of this work.[19,21] Iterative cycles of design, enzymatic tests on heparanase recombinant enzyme, and in vitro cellular assays have been implemented to obtain valuable information for drug design within the hit-to-lead discovery process. On base of dose causing 50% inhibition of heparanase enzymatic activity, as determined from dose response curves (mean of duplicates; standard deviation [SD] always <10%), RDS3333, RDS3298, RDS3337, and RDS3098 have been selected for the present work. RDS3333, RDS3298, RDS3337, and RDS3098 correspond to 13a (IC_{50} = 0.64 μM), 7b (IC_{50} = 0.37 μM), 7g (IC_{50} = 0.08 μM), and 5c (IC_{50} = 0.18 μM), respectively, as reported following the numbering of the references 19 and 21. Compounds needed for the experiments have been resynthesized as reported[19,21] obtained with the appropriate purity (>95%) and supplied in amount sufficient for the assays of this work.

The compounds RDS3333, RDS3298, RDS3337, and RDS3098 were dissolved in dimethyl sulfoxide (DMSO, Sigma-Aldrich) at the 10-mM stock solution. Cells were seeded into 6-well cell culture plates at the concentration of 2×10^5 cells/mL and maintained for 24 h at 37°C with 5% CO_2. Then, cells were treated with different concentrations of compounds (80, 320, 1280 nM) for an incubation time of 24, 48, or 72 h. Vehicle-treated cells or cells incubated with each of the four compounds were analyzed by Trypan Blue (Sigma-Aldrich) assay to evaluate cell viability.[22] DMSO is the vehicle to dissolve the compounds and we consider cells without any treatment, with only DMSO, as vehicle-treated cells.

2.2 | Purification of anti-β2-GPI antibodies

Isolation of human anti-β2-GPI antibodies was obtained by affinity chromatography, as previously reported,[8] from three APS patients (positive for anti-β2-GPI antibodies by ELISA). The three APS patients were women (ages 42, 42, and 44 years) with deep venous and arterial thromboses who had been diagnosed according to the Sidney Classification Criteria.[2] Antibody reactivity to β2-GPI was checked by western blot. The purified antibodies recognize the DI domain of β2-GPI. They showed abnormal values in at least two clotting tests that returned to normal values on confirmatory testing, displaying LAC activity. In all performed tests, the stimulatory effect of the three anti-β2-GPI antibodies was virtually the same (data not shown). As a control, we used IgG from human normal serum (Sigma-Aldrich).

2.3 | Platelet separation

Blood samples were obtained from six healthy donors that gave written informed consent from Transfusional Center of Policlinico, Sapienza University of Rome.

For platelet separation, samples in the presence of sodium citrate as anticoagulant were centrifuged at 150 g for 15 min at 20°C to obtain platelet-rich plasma (PRP). Two thirds of the PRP were drawn, without disturbing the buffy coat layer, to prevent contamination. PRP, transferred into another new sterile tube, was mixed with acid citrate dextrose (ACD) to avoid platelet activation, and centrifuged at 900 g for 10 min at 20°C (with no brake applied).[23]

After discard platelet-poor plasma (PPP), platelet pellets were resuspended with calcium-free Tyrode's buffer, containing 10% (v:v) ACD and washed as above. Then, platelets were resuspended in calcium-free Tyrode's buffer with the addition of bovine serum albumin (BSA, 3 mg/ml), previously tested as β2-GPI-free by high-performance liquid chromatography (HPLC).

A hemocytometer (Coulter, Beckman Coulter) was used to count the platelets, which revealed that leukocyte contamination was <1 leukocyte/10^7 platelets. Then, the purity of the isolated platelets was verified and confirmed by staining with a fluorescein isothiocyanate (FITC)-conjugated anti-CD41 or anti-CD61 antibody (Beckman Coulter) and analyzed by flow cytometry (Coulter Epics, Beckman Coulter; data not shown).

2.4 | In vitro incubation of endothelial cells and human platelets with anti-β2-GPI in the presence of heparanase inhibitor

For in vitro studies, HUVECs (5×10^5/mL), HMEC-1 (American Type Culture Collection, ATCC; 5×10^5/mL, see supporting information) and human platelets (3×10^8/mL) were seeded into 6-well cell culture and incubated at 37°C, for different incubation times, with affinity-purified or polyclonal anti-β2-GPI antibodies (200 μg/ml), normal human serum

IgG (200 µg/ml), or lipopolysaccharide (LPS) (100 ng/ml), according to the methods previously described.[7,24] To exclude the possibility of LPS contamination, samples were stimulated in the presence or absence of anti-β2GPI antibody and then pretreated with polymyxin B (10 µg/ml; Sigma-Aldrich). In parallel experiments, HUVECs, as well as human platelets, were pretreated with selective heparanase inhibitor RDS3337 (320 nM) for 1 h before treatments. Virtually no heparanase activity was detected in HUVECs and platelets pre-incubated with RDS3337, as detected by Heparanase Assay kit (Amsbio). On the contrary, affinity-purified as well as LPS induced a significant increase of heparanase activity and release (Figure S1 in supporting information).

All materials contained <0.00025 ng of endotoxin/µg of protein, as determined by the Limulus amebocyte lysate test (Associates of Cape Cod).

2.5 | Preparation of cell extracts

Untreated or treated HUVECs and HMEC-1 with affinity-purified anti-β2-GPI antibodies, polyclonal anti-β2-GPI antibodies, normal human serum IgG or LPS, and alternatively pretreated with heparanase inhibitor RDS3337, were incubated for different incubation times, at 37°C, in 5% CO_2. After treatments the medium was removed, cells placed on ice, washed once in phosphate-buffered saline (PBS), and scraped in PBS. To prepare whole-cell extracts, cells were resuspended in lysis buffer, containing 20 mM HEPES, pH 7.2; 1% Nonidet P-40, 10% glycerol, 50 mM NaF, 1 mM Na_3VO_4 and protease inhibitors cocktail (Sigma-Aldrich). Soluble proteins were recovered after centrifugation of lysates at 15,000 g for 15 min at 4°C. Nuclear extracts were prepared as previously described.[25] Briefly, cells were resuspended in buffer A (20 mM HEPES, pH 7.2; 0.1% Nonidet P-40, 20 mM KCl, 3.0 mM $MgCl_2$, 1 mM Na_3VO_4, 5 mM DTT, and protease inhibitors cocktail), after 30 min on ice, and then centrifuged for 30 min at 10,000 g at 4°C. Pellets were resuspended in buffer B (40 mM HEPES, pH 7.2; 0.84 M NaCl, 0.4 mM EDTA, 50% glycerol, 1 mM Na_3VO_4, 5 mM DTT, and protease inhibitors cocktail), after 1 h on ice, samples were centrifuged at 10,000 g for 1 h at 4°C and supernatants (nuclear extracts) were transferred to new vials.

Human platelets, unstimulated or stimulated with either affinity-purified or polyclonal anti-β2-GPI antibodies, normal human serum IgG, or LPS, and alternatively pretreated with heparanase inhibitor RDS3337, were incubated for different incubation times at 37°C in 5% CO_2. Then, samples were resuspended in lysis buffer and whole-cell extracts were obtained as described above.

Protein content was determined by Bradford assay using BSA as a standard (Bio-Rad). Samples were frozen at −80°C.

2.6 | Western blot analysis of phospho-IRAK1 and phospho-NF-κB

Equal amounts of whole (40 µg/sample) or nuclear (20 µg/sample) extract proteins of HUVECs or HMEC-1 and whole extracts proteins (40 µg/sample) of human platelets, untreated or treated with either affinity-purified or polyclonal anti-β2-GPI antibodies, normal human serum IgG, or LPS (HUVECs for 45 min and platelets for 10 min), and alternatively pretreated with heparanase inhibitor RDS3337 for 1 h, were separated in 7.5% sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE) under unreducing conditions. Proteins were electrophoretically transferred to polyvinylidene difluoride (PVDF) membranes (Bio-Rad Laboratories) and then, after blocking with Tris-buffered saline Tween 20 (TBS-T) 3% BSA, incubated with polyclonal rabbit anti-phospho-IRAK1 (Cell Signaling, Inc.) or polyclonal rabbit anti-phospho-NF-κB-p65 antibodies (Cell Signaling, Inc.). Antibody reactions were visualized by horseradish peroxidase (HRP)-conjugated anti-rabbit IgG (Sigma-Aldrich), and then by the chemiluminescence reaction using enhanced chemiluminescence (ECL) western blot system (Amersham Pharmacia Biotech).

To adjust for total protein content, phospho-IRAK1-blotted membranes were stripped and reprobed with polyclonal anti-IRAK1 antibody (MBL) or anti-β-actin mAb (Sigma-Aldrich). As a control for loading and purity of preparation, phospho-NF-κB-p65-blotted membranes of HUVECs and HMEC-1 were reprobed with polyclonal anti-histone H1 antibodies (Abcam) whereas for loading control, phospho-NF-κB-p65-blotted membranes of human platelets were stripped and reprobed with anti-NF-κB-p65 (Cell Signaling, Inc.) or with anti-β-actin mAb (Sigma-Aldrich).

Densitometric scanning analysis was performed by NIH Image 1.62 software (National Institutes of Health). The density of each band (absolute value) in the same gel was analyzed.

2.7 | Analysis of TF expression

Preliminarily, TF expression on platelets was analyzed by immunofluorescence. Unstimulated human platelets or stimulated with affinity-purified anti-β2-GPI antibodies for 45 min at 37°C were dried-fixed on glass slides. Then, platelets were fixed with 4% paraformaldehyde in PBS for 30 min at room temperature and then permeabilized by 0.5% Triton X-100 in PBS for 5 min at room temperature. After washing nonspecific binding sites were blocked with PBS containing 3% BSA. Platelet CD61 and TF were immunolabeled with FITC-conjugated anti-CD61 mAb (Beckman Coulter) or with FITC-conjugated anti-TF mAb (Sino Biological). The slides were examined by fluorescence microscopy. The images were acquired and observed with an Olympus BX51 fluorescence microscope (Olympus Italia S.r.l.; ×63 magnification) equipped with an F- View digital camera and the Cell-F Digital Imaging Software.

In addition, modulation of TF expression was analyzed by western blot analysis. Equal amounts of proteins lysates (40 µg/sample) of HUVECs and human platelets (from untreated or treated with affinity-purified anti-β2-GPI antibodies, normal human serum IgG, or LPS for 4 h, and alternatively pretreated with heparanase inhibitor RDS3337 for 1 h) were subjected to SDS-PAGE on 10% gels and then electrophoretically transferred to PVDF membranes. The

membranes were blocked with TBS-T containing 3% BSA and subsequently probed with rabbit anti-TF mAb (Abcam). Bound antibodies were visualized with HRP-conjugated anti-mouse IgG, and immunoreactivity was assessed by ECL reaction using the ECL western blot system. For loading control TF-blotted membranes were reprobed with anti-β-actin mAb (Sigma-Aldrich).

2.8 | Analysis of TF procoagulant activity

A two-stage factor X-activating assay[26] was used to analyze the procoagulant activity of TF released in supernatant of platelets treated with affinity-purified anti-β2-GPI antibodies (200 μg/ml), normal human serum IgG (200 μg/ml), or LPS (100 ng/ml), in presence or not of heparanase inhibitor RDS3337 (320 nM, 1 h before treatments). Briefly, platelets from normal donors were stimulated in TBS containing 0.1% BSA and supernatants were collected. A fixed amount of proteins (100 μg) released from platelets was incubated in triplicate with activated factor VII (FVIIa; 10 ng) and $CaCl_2$ (5 mM) for 15 min at 37°C. Then we added human factor X (250 ng) and the mixture was reincubated for 30 min at 37°C. Finally, 10 μl of FXa-specific chromogenic substrate (5 mM) was added and factor Xa activity was quantitated by measuring the rate of increase in absorbance at 405 nm using an ELISA plate reader. All coagulation factors were purchased from Sigma-Aldrich.

2.9 | Platelet activation

Platelets from healthy donors were obtained as reported above. Platelets were treated with a mix of agonists composed by U-446619 (1 μM; synthetic thromboxane A2 receptor agonist, Helena Biosciences Europe) plus epinephrine (10 μM; Helena Biosciences Europe) or affinity-purified anti-β2-GPI antibodies (200 μg/ml), and alternatively pretreated with heparanase inhibitor RDS3337 for 1 h, were analyzed to evaluate platelet aggregation. Briefly, platelets were added, after treatment, in appropriate wells and the plate, covered with an acrylic adhesive thin layer, was placed immediately in a Plate Reader Victor 3 (PerkinElmer). The absorbance, carrying out an orbital shaking for 10 s, before each reading, was determined at 590 nm every 60 s at 37°C. The platelet aggregation percentage (PA%) was assessed using the following formula PA% = (sample UA absorbance-PRP UA absorbance)/(PPP UA absorbance-PRP UA absorbance) × 100.

Platelet function was also investigated evaluating platelet secretion by ATP release dosage. ATP release, post-platelet activation, was quantified using a luciferin/luciferase method (ATP lite, PerkinElmer), as ATP monitoring system. This method was performed as reported.[27] Briefly, ATPlite, according to the manufacturer's instructions, was added to the platelet suspension immediately after the treatments. The plate was placed in a Plate Reader Victor 3 (PerkinElmer) to measure the emitted light every 60 s at 37°C for 10 min. The results were analyzed as percentage of maximal secretion compared to total intraplatelets adenyl nucleotides content.

2.10 | Statistical analysis

Data obtained are expressed as means ± SD of at least six independent experiments. Statistical analysis was performed with two statistical tests. To compare differences between percentages of Trypan Blue positive cells we used Chi-square (χ^2) tests. In the other experiments the paired Student's t-test was used. Statistical significance was set at $P \leq .01$; P values >.01 were not significant (NS).

3 | RESULTS

3.1 | Preliminary analysis of activity and cytotoxic effect of Heparanase inhibitors

Four newly synthesized compounds were already tested for their ability to inhibit heparanase activity using an in vitro assay.[19,21] The compound RDS3337 (Figure 1) revealed the highest anti-heparanase activity among the tested compounds, showing nanomolar potency. Indeed, it reported an IC_{50} value of 0.08 μM, being therefore about 2–8 more potent than its analogues (Table 1). These compounds were further studied to assess their effect on cell viability. Thus, we performed a preliminary analysis to exclude their cytotoxic effect, under
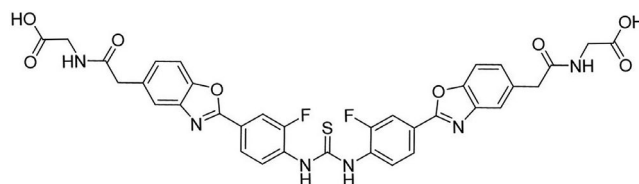

FIGURE 1 Newly synthesized compound RDS3337 that reports anti-heparanase activity

TABLE 1 Heparanase inhibitory activities of the newly synthesized compounds RDS3333, RDS3298, RDS3337, and RDS3098.

Compound	IC_{50} (μM)[a]
RDS3333 (13a)	0.64
RDS3298 (7b)	0.37
RDS3337 (7g)	0.08
RDS3098 (5c)	0.18

[a]Dose causing 50% inhibition of heparanase enzymatic activity as determined from dose response curves (mean of duplicates; standard deviation always < 10%) repeated at least twice in separate experiments, stated in micromole concentration.

our experimental conditions, using Trypan Blue assay. HUVECs were treated with RDS3333, RDS3298, RDS3337, and RDS3098 (concentration range 80–1280 nM) for different incubation times (24–72 h) and then analyzed by cell counting (Figure 2). The results indicate that RDS3337 is the most interesting compound; this derivative proved to be the most active to inhibit heparanase enzyme, interfering least with cell viability. Thus, we decided to deepen the role of compound RDS3337, as selective heparanase inhibitor, in biological activity studies.

3.2 | Heparanase inhibitor RDS3337 decreases endothelial cell activation signaling triggered by anti-β2-GPI antibodies

Because heparanase may play a role in endothelial dysfunction, we investigated whether the selective heparanase inhibitor RDS3337 is able to affect the activation of molecules usually involved in a proinflammatory signaling. Several data prove that anti-β2-GPI antibodies can activate endothelial cells leading to a proinflammatory phenotype; thus, we analyzed by western blot IRAK phosphorylation and NF-κB activation. Analysis of cell lysates from HUVECs showed that affinity-purified anti-β2-GPI antibodies, as well as LPS, induced IRAK1 phosphorylation (Figure 3A) and NF-κB-p65 activation (Figure 3B), as revealed, respectively, by anti-phospho-IRAK1 and anti-phospho-NF-κB-p65 antibodies' reactivity. Virtually no activation was obtained in untreated cells or following treatment with control human IgG. On the contrary, IRAK phosphorylation and NF-κB-p65 activation significantly decreased when cells were pretreated with the heparanase inhibitor RDS3337 (Figure 3A,B). Similar findings were obtained after triggering of HMEC-1 with polyclonal anti-β2-GPI antibodies (Figure S2 in supporting information).

These data support the view of heparanase involvement in endothelial cell activation.

3.3 | Heparanase inhibitor RDS3337 decreases platelet activation signaling triggered by anti-β2-GPI antibodies

It is well known that platelets may play a role in thrombotic manifestations by binding of aPL, and are a rich source of heparanase. Starting from these data, we analyzed whether the heparanase inhibitor can prevent platelet activation and the signal transduction pathway triggered by anti-β2-GPI antibodies. We analyzed by western blot IRAK phosphorylation and NF-κB activation in platelet lysates. As expected, the treatment with affinity-purified anti-β2-GPI antibodies, as well as LPS, induced an increase of phospho-IRAK1 (Figure 4A) and phospho-NF-κB-p65 expression (Figure 4B) compared to untreated platelets or treated with control human IgG. Interestingly, when platelets were pretreated with the selective heparanase inhibitor RDS3337, both anti-phospho-IRAK1 and anti-phospho-NF-κB-p65 reactivity were significantly inhibited (Figure 4A,B). Similar findings were obtained after triggering with polyclonal anti-β2-GPI antibodies (Figure S2), demonstrating a functional role of heparanase enzyme in platelet activity.

3.4 | Heparanase inhibitor RDS3337 decreases TF expression triggered by anti-β2-GPI antibodies

We analyzed preliminary expression and distribution of TF in platelets (Figure 5). Immunofluorescence analysis revealed an uneven distribution of the staining mainly in the cytoplasm. Following treatment with affinity-purified anti-β2-GPI antibodies, TF staining was increased and appeared also localized on cell plasma membrane (see arrows).

Thus, we analyzed the expression of TF as a procoagulant factor not only in endothelial cells, but also in platelets and investigated the role of the heparanase inhibitor RDS3337 on TF expression.
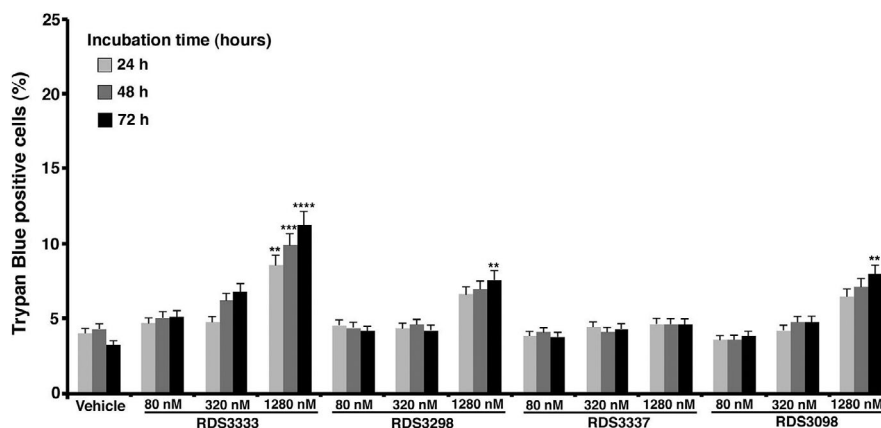

FIGURE 2 Effect on cell viability of newly synthesized compounds RDS3333, RDS3298, RDS3337, and RDS3098. Human umbilical vein endothelial cells were cultured with three different concentrations of the compounds (80, 320, and 1280 nM) for 24, 48, and 72 h. The number of viable cells was determined by Trypan Blue exclusion test. Data are reported as the mean ± standard deviation (SD) among 10 independent experiments. Statistical analysis: **$P < .01$ versus vehicle, ***$P < .001$ versus vehicle, ****$P < .0001$ versus vehicle. RDS3337 treated samples versus vehicle NS, not significant
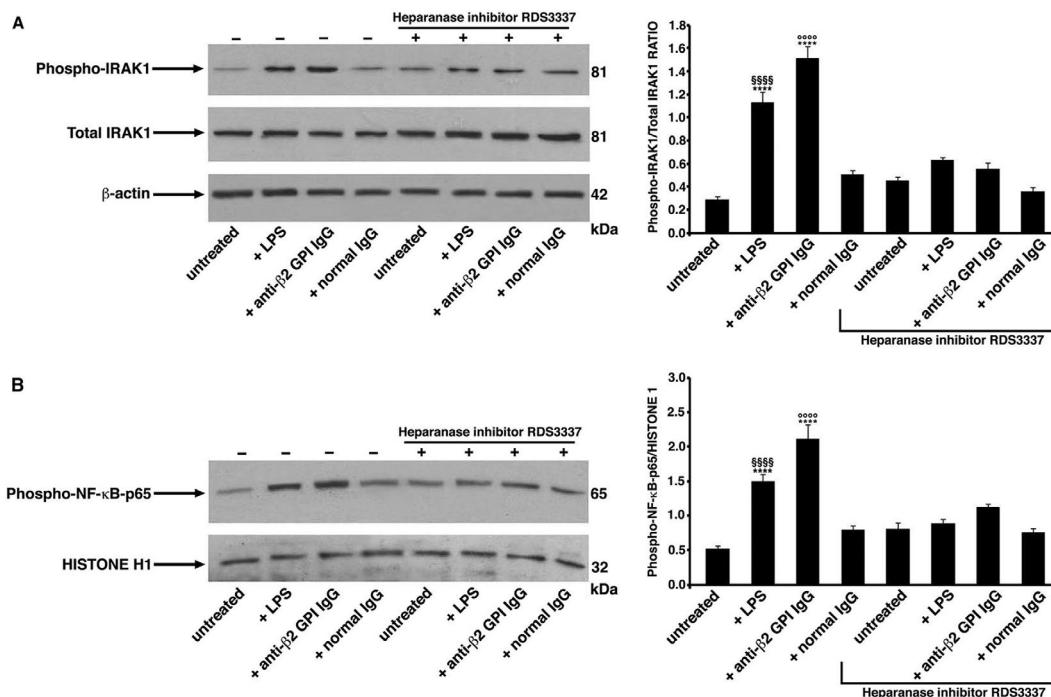
FIGURE 3 Heparanase inhibitor RDS3337 reduces interleukin-1 receptor-associated kinase 1 (IRAK) phosphorylation and nuclear factor kappa B (NF-κB) activation triggered by anti-β2-glycoprotein I (β2-GPI) antibodies in endothelial cells. Human umbilical vein endothelial cells were treated for 45 min with affinity-purified anti-β2-GPI antibodies (200 μg/ml) and, as controls, with lipopolysaccharide (LPS; 100 ng/ml) or control human IgG (200 μg/ml). Alternatively, cells were pretreated with heparanase inhibitor RDS3337 (320 nM) for 1 h. After treatments cells were analyzed by western blot for IRAK phosphorylation and NF-κB-p65 activation. A, Phosphorylated levels of IRAK1 were evaluated in whole cell extracts using rabbit anti-phospho-IRAK1 Ab. The membrane was stripped and reprobed with polyclonal anti-IRAK1 Ab. For loading control was used anti-β-actin mAb. Densitometric phospho-IRAK1/Total IRAK1 ratios are shown in the right panel. Results represent the mean ± SD from six independent experiments. Statistical analysis indicates: ****$P < .0001$ versus untreated; §§§§$P < .0001$ versus RDS3337 + LPS; °°°°$P < .0001$ versus RDS3337 + anti-β2-GPI antibodies. B, Nuclear cell extracts were analyzed to verify phospho-NF-κB-p65 expression using rabbit anti-phospho-NF-κB-p65 Ab. As a control, for loading and purity of preparation, membrane was stripped and reprobed with polyclonal anti-HISTONE H1 Ab. Densitometric phospho-NF-κB-p65/HISTONE H1 ratios are shown in the right panel. Results represent the mean ± SD from six independent experiments. Statistical analysis indicates: ****$P < .0001$ versus untreated; §§§§$P < .0001$ versus RDS3337 + LPS; °°°°$P < .0001$ versus RDS3337 + anti-β2-GPI antibodies

As shown in Figure 5, western blot analysis of endothelial cells and platelet lysates revealed an increase of TF expression following incubation with affinity-purified anti-β2-GPI antibodies or LPS, which was reported to induce a significant increase of TF synthesis[24] as well as of TF expression.[28] Conversely, virtually no reactivity was observed in untreated cells or in cells stimulated with control human IgG. Furthermore, in samples pretreated with the heparanase inhibitor RDS3337, we obtained a significant decrease of TF levels (Figure 6A,B).

In parallel experiments procoagulant activity of TF was evaluated. As expected, we observed a significant increase ($P < .001$) of TF activity released from platelets incubated with affinity-purified anti-β2-GPI antibodies compared to control cells (OD 0.79 + 0.025 vs. 0.40 + 0.035), which was partially prevented by previous pretreatment with the heparanase inhibitor RDS3337 (OD 0.45 + 0.019).

These results suggest an implication of heparanase in determining a prothrombotic state related to endothelial cell injury and platelet activation.

3.5 | Heparanase inhibitor RDS3337 decreases platelet aggregation and ATP release triggered by anti-β2-GPI antibodies

To investigate the effect of heparanase inhibitor on functional platelet activation, platelets from healthy donors were treated with anti-β2-GPI antibodies. As shown in Figure 7, an increase of platelet aggregation following incubation with affinity-purified anti-β2-GPI antibodies was detected, which was decreased in samples pretreated with the heparanase inhibitor RDS3337 (64.4%).

Moreover, to deepen platelet activation, the secretion of adenyl nucleotides was measured. Results highlighted an increase of ATP release in samples stimulated with anti-β2-GPI antibodies, which was partially prevented by previous pretreatment with the heparanase inhibitor RDS3337 (57.1%).

Alternatively, for both tests (aggregation and ATP release), platelets were treated with a mix of control agonists composed by U-446619 plus epinephrine. In this case, virtually no reduction was observed when cells were pretreated with the heparanase inhibitor
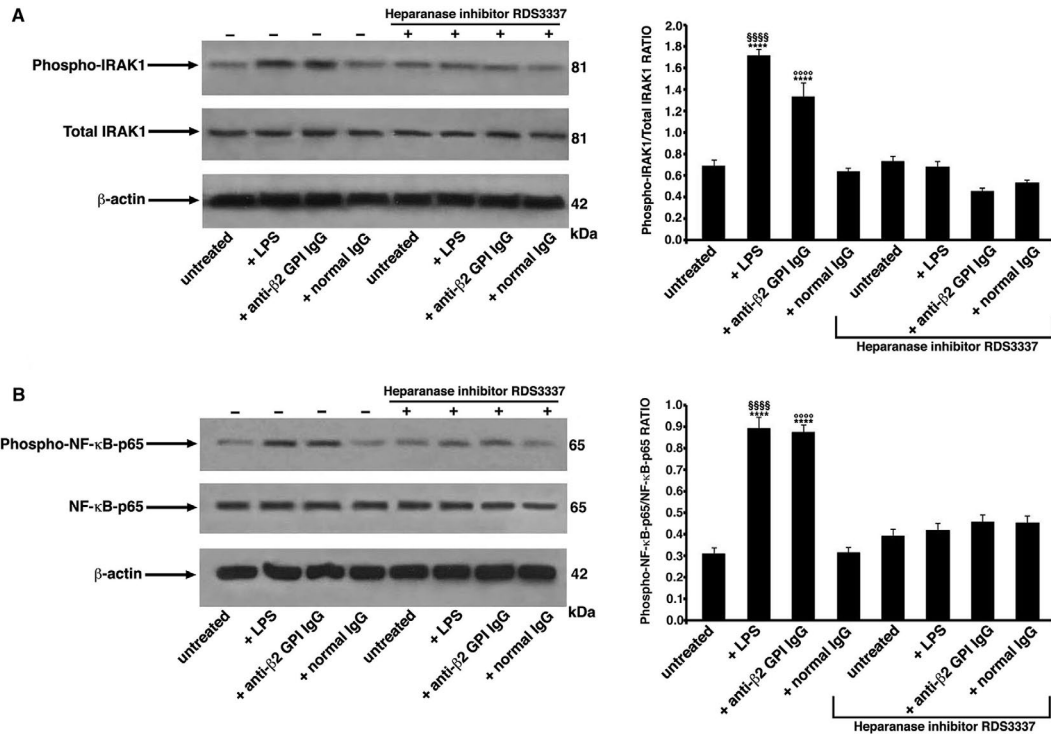
FIGURE 4 Heparanase inhibitor RDS3337 decreases interleukin-1 receptor-associated kinase 1 (IRAK) phosphorylation and nuclear factor kappa B (NF-κB) activation triggered by anti-β2-glycoprotein I (β2-GPI) antibodies in platelets. Human platelets from healthy donors were treated for 10 min with affinity-purified anti-β2-GPI antibodies (200 μg/ml) and, as controls, with lipopolysaccharide (LPS; 100 ng/ml) or control human IgG (200 μg/ml). Alternatively, cells were pretreated with heparanase inhibitor RDS3337 (320 nM) for 1 h. After treatments cells were analyzed by western blot for IRAK phosphorylation and NF-κB-p65 activation. A, Whole platelet extracts were used to determine the levels of phospho-IRAK1 expression using rabbit anti-phospho-IRAK1 Ab. The membrane was stripped and reprobed with polyclonal anti-IRAK1 Ab. For loading control was used anti-β-actin mAb. Densitometric phospho-IRAK1/Total IRAK1 ratios are shown in the right panel. Results represent the mean ± standard deviation (SD) from six independent experiments. Statistical analysis indicates: ****$P < .0001$ versus untreated; §§§§$P < .0001$ versus RDS3337 + LPS; °°°°$P < .0001$ versus RDS3337 + anti-β2-GPI antibodies. B, Phosphorylated levels of NF-κB-p65 were evaluated in whole platelet extracts using anti-phospho-NF-κB-p65 Ab. The membrane was stripped and reprobed with polyclonal anti-NF-κB-p65 Ab. For loading control was used anti-β-actin mAb. Densitometric phospho-NF-κB-p65/NF-κB-p65 ratios are shown in the right panel. Results represent the mean ± SD from six independent experiments. Statistical analysis indicates: ****$P < .0001$ versus untreated; §§§§$P < .0001$ versus RDS3337 + LPS; °°°°$P < .0001$ versus RDS3337 + anti-β2-GPI antibodies
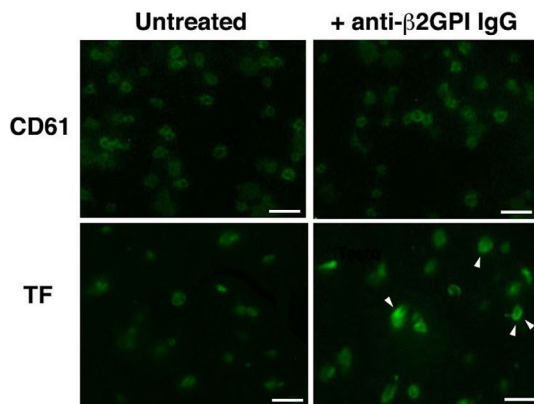

FIGURE 5 Platelet TF expression and distribution. Untreated human platelets and treated with anti-β2-glycoprotein I (β2-GPI) for 45 min at 37° were fixed with 4% paraformaldehyde and then permeabilized with 0.5% Triton X-100. The immunofluorescence pattern of platelets incubated with FITC-conjugated anti-CD61 mAb or with FITC-conjugated anti-TF mAb is shown. Scale bars: 20 μm

RDS3337, suggesting that the inhibitory effect of RDS3337 is quite specific.

4 | DISCUSSION

This study demonstrates that platelet and endothelial cell TF expression induced by human anti-β2-GPI antibodies is prevented by heparanase inhibitor. We tested a newly synthesized compound (RDS3337), which was revealed to be highly active without interfering with cell viability. This compound interacts with HBD-1 and HBD-2 regions of heparanase, thus undertaking a high number of polar contacts with the protein counterpart. According to Messore et al.[19] RDS3337 (7g) may be considered highly specific for heparanase, although we cannot exclude other off-target effects. Moreover, our results showed a significant inhibitory effect on the signal transduction pathway(s) triggered by anti-β2-GPI antibodies.
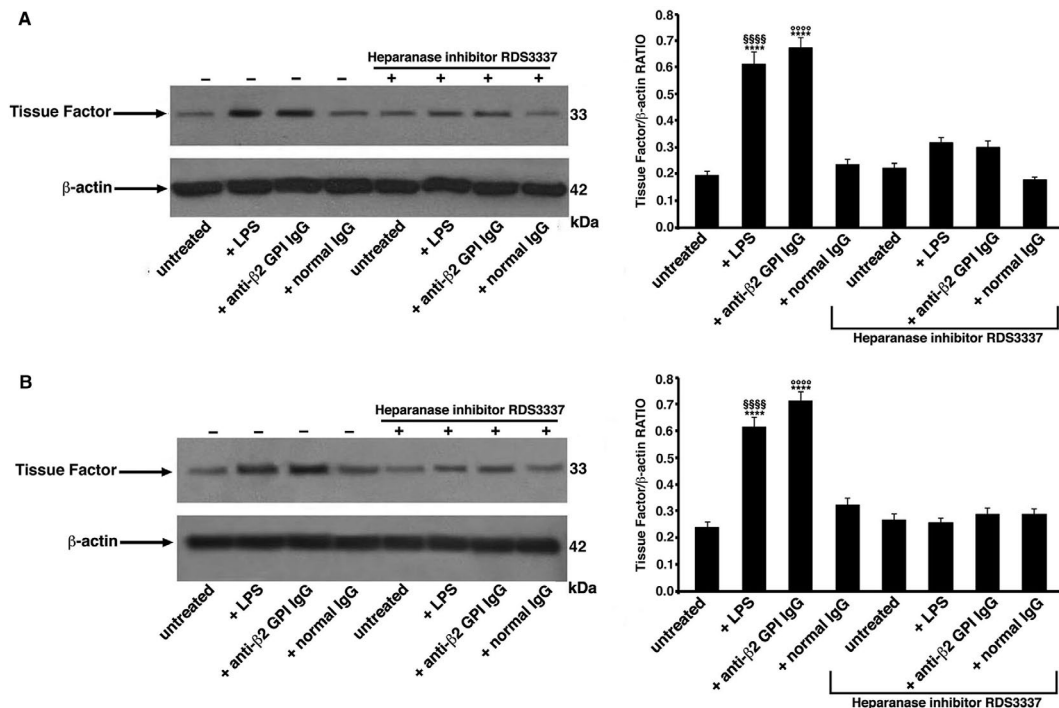
FIGURE 6 Heparanase inhibitor RDS3337 reduces tissue factor (TF) expression triggered by anti-β2-glycoprotein I (β2-GPI) antibodies in endothelial cells and platelets. Human umbilical vein endothelial cells and human platelets from healthy donors were treated for 4 h with affinity-purified anti-β2-GPI antibodies (200 μg/ml) and, as controls, with lipopolysaccharide (LPS; 100 ng/ml) or control human IgG (200 μg/ml). Alternatively, cells were pretreated with heparanase inhibitor RDS3337 (320 nM) for 1 h. After treatments, whole extracts of endothelial cells (A) and platelets (B) were analyzed by western blot to determine TF expression using rabbit anti-TF mAb. For loading control, membrane was stripped and reprobed with anti-β-actin mAb. Densitometric TF/β-actin ratios are shown in the right panel. Results represent the mean ± standard deviation (SD) from six independent experiments. Statistical analysis indicates: (A) ****P < .0001 versus untreated; §§§§P < .0001 versus RDS3337 + LPS; °°°°P < .0001 versus RDS3337 + anti-β2-GPI antibodies. (B) ****P < .0001 versus untreated; §§§§P < .0001 versus RDS3337 + LPS; °°°°P < .001 versus RDS3337 + anti-β2-GPI antibodies
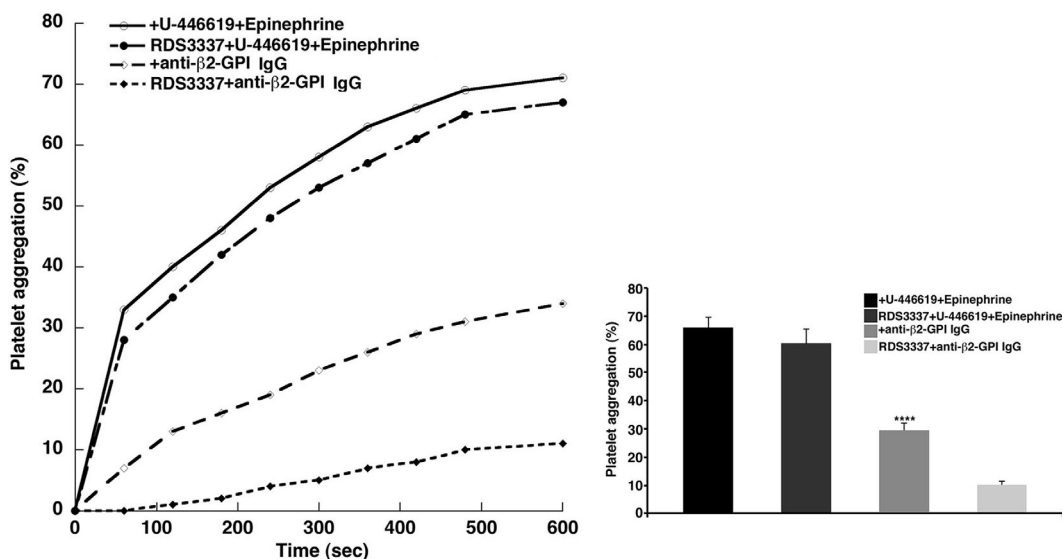

FIGURE 7 Heparanase inhibitor RDS3337 decreases platelet aggregation. Human platelets from healthy donors were treated with a mix of agonists composed by 1 μM U-446619 plus 10 μM epinephrine or with affinity-purified anti-β2-glycoprotein I (β2-GPI) antibodies (200 μg/ml). Alternatively, cells were pretreated with heparanase inhibitor RDS3337 (320 nM) for 1 h. After treatments platelets were analyzed, by a Plate Reader Victor 3, to evaluate platelet aggregation. Results are representative of six independent experiments. Statistical analysis indicates: ****P < .0001 versus RDS3337 + anti-β2-GPI antibodies

In previous studies we demonstrated that these antibodies are able to trigger IRAK phosphorylation and NF-κB translocation, leading to a pro-inflammatory and pro-coagulant monocyte phenotype, characterized by overexpression and release of TF.[8] Similar findings were also observed in endothelial cells.[29] Several mechanisms have been proposed for endothelial cell activation by aPL. It may result from the direct binding of β2-GPI to endothelial cells and the activation of inflammatory receptors on these cells.[30] Similarly, Laplante et al.[31] showed in a carotid artery injury model that anti-β2-GPI activation of endothelial cells is dependent on TLR-4. Conversely, anti-β2-GPI antibodies enhance the production of pro-thrombotic and pro-inflammatory responses in blood vessels. Moreover, antibodies may activate endothelial cells increasing the expression level of von Willebrand factor in their surface, which can contribute to platelet activation. Indeed, recently we reported that anti-β2-GPI antibodies also induce TF expression in platelets.[7] The presence of TLR-4 in platelets is currently well known and may play a role in thrombocytopenia induced by LPS.[32] Indeed, platelets contain all of the proteins (e.g., MyD88, IRAK, and interferon regulatory factor 3 [IRF3]), that are required for signal transduction through TLR-4 and utilize the same mechanisms as in nucleated cells.[33] Thus, anti-β2-GPI triggered activation of the TLR-4 signaling pathway in platelets may play a role in some pathological manifestations of APS. Indeed, TLR-4 is able to trigger a signaling cascade leading to the activation of transcription factors through both MyD88-IRAK–dependent and-independent pathways, with consequent functional effect on platelet activity, hemostasis, and thrombosis.[34] Moreover, our results showed a significant increase of TF in platelets from APS patients compared to control subjects.[7]

In the present study we confirmed and extended these findings, demonstrating for the first time the *in vitro* effect of heparanase inhibitors on both TF expression on platelets and endothelial cells and on the signal transduction pathway triggered by anti-β2-GPI antibodies. The same effect was observed on the signaling triggered by LPS; this observation is not surprising, as it was well known that heparanase activation induces TF expression.[17] We cannot exclude the possibility that heparanase fragments may induce TF expression on the cells. Anyway, our findings suggest that heparanase activity is released very shortly after anti-β2-GPI antibody stimulation. Thus, an interplay between heparanase inhibitor and TLR-4 could be hypothesized. However, recent data demonstrated that heparanase-induced cytokine release was abolished by enzymatic-inhibitors of heparanase. Because soluble heparanase can signal through the TLR pathway, heparanase may also promote upregulation of cytokines through the generation of heparanase-cleaved fragments of the enzyme. The pathway involved in cytokine upregulation was identified as NF-κB-dependent.[16]

Heparanase activity is implicated in neovascularization, inflammation, and autoimmunity. Heparanase expression is enhanced in patients with Crohn's disease and ulcerative colitis and in the synovial fluid and tissue of patients with rheumatoid arthritis. It has also been implicated in the severity of atherosclerosis, because its expression is upregulated in vulnerable coronary plaques.[14,35-37] This enzyme can play a role as inflammatory mediator, modifying and destroying endothelial surface layer structure, leading to the impairment of endothelial functions, promoting progression of vascular diseases.[38] Moreover, heparanase induces the transcription of pro-angiogenic, pro-thrombotic, and pro-inflammatory factors, promoting the release of pro-inflammatory cytokines from human peripheral blood mononuclear cells and generating soluble HS fragments that activate TLR-dependent pathway(s). In fact, cytokine induction by heparanase appears to involve TLR-2, TLR-4, and NF-κB.[16,17,39] The involvement of the enzyme in inflammatory reactions was further supported by anti-inflammatory effects demonstrated for heparanase-inhibiting substances (i.e., heparin, synthetic heparin-mimicking compounds) in animal and clinical studies. Thus, therapeutic benefits are expected in inflammation by pharmacological strategies that target heparanase, reducing heterotypic interactions among epithelial, endothelial, and immune cells due to the enzyme involvement.[40-42]

Heparanase upregulates the expression of TF and interacts with the TF pathway inhibitor (TFPI) on the cell surface membrane of endothelial cells, leading to dissociation of TFPI and increasing coagulation activity. It was also demonstrated that heparanase overexpressing mice generated a larger thrombus within a shorter period of time compared to control mice, suggesting the procoagulant effect of heparanase.[18,43] In conclusion, demonstration that this new synthesized heparanase inhibitor (RDS3337) is able to prevent the main signal transduction pathway triggered by anti-β2-GPI antibodies in endothelial cells and platelets introduces a new task in the pharmacological approach of APS. In addition, heparanase activity detection may be useful in monitoring patients and evaluating risk stratification. Further studies are in progress to test the levels of heparanase activity in APS patients and the relationship with disease progression.

CONFLICTS OF INTEREST

The authors declare no conflicts of interest.

AUTHOR CONTRIBUTIONS

M.S., R.D.S., and A.L. conceived and designed the study. G.R. and S.R. performed the experiment; A.C., V.M., and F.S. analyzed the data; A.C., R.M., A.L., and M.S. wrote the original manuscript. M.S., T.G., R.C., and F.P. read and approved the final version of the manuscript.

ETHICAL APPROVAL

The study was conducted in compliance with the Declaration of Helsinki and the local ethical committee approved this study.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.