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Identification of the binding site for fondaparinux on Beta2-glycoprotein I

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Abstract

Antiphospholipid syndrome (APS) is an autoimmune disease with clinical manifestations of thrombosis and pregnancy complications. Beta2-glycoprotein I (2GPI) is the major antigen for the APS-related antibodies. Heparin, low molecular weight heparin and the synthetic pentasaccharide fondaparinux are commonly used for prophylaxis and treatment of thrombosis in patients with antiphospholipid syndrome. These antithrombotic drugs bind and activate antithrombin III to inactivate blood clotting proteases. Heparin and heparin derivatives might have a direct beneficial effect in APS via binding to 2GPI and interfering with prothrombotic properties of 2GPI/antibody complexes. We compared fondaparinux to heparin regarding its ability to bind 2GPI and inhibit the binding of 2GPI/antibody complexes to negatively charged phospholipids and endothelial cells. Although heparin and fondaparinux bind 2GPI at therapeutically relevant doses, neither fondaparinux nor heparin was efficient in inhibition of the binding of 2GPI/antibody complexes to negatively charged phospholipids and endothelial cells. Our studies suggest that these drugs do not act on pathological properties of 2GPI/antibody complexes, emphasizing the need for a new treatment specific for 2GPI-related thrombosis in APS. We observed that the binding interface of fondaparinux on 2GPI does not include the lysine residues known to be critical for binding of heparin. The docking model of the 2GPI complex with fondaparinux is in agreement with multiple experimental observations.

Keywords

Beta2-glycoprotein I; antiphospholipid syndrome; NMR; molecular docking; thrombosis; heparin binding protein

1. Introduction

Antiphospholipid syndrome (APS) is a disease in which the presence of autoimmune antibodies detected by laboratory tests correlates with the clinical features of thrombosis and/or pregnancy complications [1]. Antiphospholipid antibodies directed to beta2-glycoprotein I (2GPI) associate the most with thrombotic events in APS patients [2,3].

2GPI is one of the most abundant proteins in the blood, with a concentration of about 4 μ M. It consists of five domains and can adopt two conformations: circular, where the first

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domain comes close to domain five [4] and extended [5,6]. In the blood, majority of 2GPI molecules circulate in a circular conformation. Binding to anionic phospholipids converts 2GPI from closed to open conformation, fully exposing epitopes for pathological anti-2GPI antibodies and, possibly, new binding sites for cellular receptors [4,7,8]. Initial binding of 2GPI to anionic phospholipids is stabilized by anti-2GPI antibodies, which enhance the binding of 2GPI to membranes more than ten folds [4,9,10]. Each of the five domains of 2GPI has two disulfide bonds. In addition to two stable disulfide bonds, domain five has a labile disulfide bond that could be easily reduced. It was suggested that the oxidative state of 2GPI could play a role in the pathophysiology of antiphospholipid syndrome [11,12].

How the presence of autoimmune anti-2GPI antibodies leads to thrombosis is not completely understood. One of the proposed prothrombotic mechanisms of the 2GPI/anti-2GPI antibody complexes is interference with the anticoagulant properties of annexin V [13]. The 2GPI/anti-2GPI antibody complexes bound to anionic phospholipids disrupt the ordered protective shield formed by annexin V on negatively charged phospholipids exposed on the surfaces of activated cells. It was demonstrated both in vitro and in vivo that the 2GPI/anti-2GPI antibody complexes activate various cells (reviewed in [8,14]). A number of cell-surface receptors were identified that bind 2GPI/anti-2GPI antibody complexes including Toll-like receptors (TLR), annexin A2, glycoprotein Ib and receptors from the LDLR family including ApoER2. Both receptors GPIba and ApoER2 are required for platelet activation [15,16]. On endothelial cells, 2GPI/anti-2GPI antibody complexes bind to a multiprotein signaling complex including TLR4, annexin A2, calreticulin and nucleolin [17]. ApoER2 mediates leukocyte adhesion to endothelial cells in the presence of anti-2GPI antibodies [18].

Presently, there is no treatment specific for 2GPI-related thrombosis in APS. Heparin, low-molecular weight heparin (LMWH) and the synthetic pentasaccharide fondaparinux that mimics the naturally occurring oligosaccharide sequence in heparin are commonly used for prophylaxis and treatment of thrombotic and obstetric complications in APS [14,19]. These antithrombotic drugs bind and activate antithrombin III to inactivate blood clotting proteases. Heparin and its derivatives bind specifically to 2GPI and, therefore, have the potential to interfere with prothrombotic mechanisms of 2GPI/antibody complexes in antiphospholipid syndrome [20]. Detailed knowledge of how heparin and its derivatives interact with 2GPI is helpful in understanding the effect of these drugs on the pathological properties of the 2GPI/anti-2GPI antibody complexes.

2. Material and methods

2.1 Proteins and reagents

Domain five of 2GPI (residues 244-326) was subcloned into a pET15b vector (Novagen). The encoded protein has an N-terminal histidine tag followed by the sequence recognized by the Tobacco Etch Virus (TEV) protease so that the tag can be removed. Domain five mutants (Met271/Leu and Met271/Leu, Lys250-251/AspAsp), A1, a fragment of mouse ApoER2 (residues 12-47 from the mature protein) and a dimeric A1-A1 molecule were expressed as TrpLE fusion proteins. The TrpLE fusion is cleaved from the protein domains by treatment with CNBr. Domain five of 2GPI, domain five mutants, A1 and A1-A1 were expressed in *E.coli*, recovered from inclusion bodies and refolded by dialysis at 4°C under conditions permitting disulfide exchange before final purification by reversed-phase HPLC on a C18 as previously described [9]. 2GPI was purchased from Haematologic Technologies, anti-2GPI antibodies were from Cedarlane, heparin and heparan sulfate were from Sigma, enoxaparin was purchased from Celsus Laboratories, fondaparinux was

purchased as a sterile injectable solution and HUVEC (HUVEC-Umbil Vein, Pooled Cells) were from Lonza.

2.2. Fluorescence spectroscopy

Binding of fondaparinux to domain five of β 2GPI was monitored by a change in protein tryptophan fluorescence. Titrations were performed with increasing concentrations of fondaparinux added to 1 μ M of domain five or 5 μ M of domain five mutants (Met271/Leu and Met271/Leu, Lys250-251/AspAsp) in 10 mM Bis-Tris, pH 7.0 buffer containing different amounts of NaCl. The total sample volume was 2.5 ml in a 1 cm quartz cuvette. Fondaparinux was added in small increments from a concentrated stock so that the resultant maximum dilution was less than 7%. Measurements were made on an AB2 Luminescence Spectrometer (Thermo Spectronic, Rochester, NY) at an excitation wavelength of 293 nm and emission wavelengths in the range of 320-360 nm with a bandpass of 8 nm. Maximum fluorescence intensity observed at 350 nm was fitted to the quadratic binding equation with K_d as the fitting parameter. Fondaparinux stock concentration was estimated from dry-weight measurement and from stoichiometric titrations of antithrombin with fondaparinux as described in [21].

2.3. Isothermal Titration Calorimetry

ITC titrations were performed at 298K using a iTC200 system from MicoCal Inc. with 20 injections of 2 μ L (first injection 0.4 μ L) of fondaparinux and a delay of 120 s between injections. The concentration of domain five of β 2GPI in a sample cell was 50 μ M and 750 μ M of fondaparinux was placed in the injection syringe. Titrations were performed in 25 mM cacodylate buffer, pH 7.2 containing 50 mM, 100 mM and 150 mM NaCl. Heat of dilution was measured by titrating 750 μ M fondaparinux into buffer and was subtracted from the integrated data prior to curve fitting.

2.4. NMR spectroscopy

All NMR experiments were conducted on a Varian Inova 500 MHz spectrometer equipped with four RF channels and a triple-resonance probe with pulsed-field gradients. Spectra were acquired at 298K using BioPack (Varian Inc.) pulse sequences and processed with GIFA v4.3 software [22]. Chemical shifts were referenced to 2,2-dimethyl-2-silapentane-5-sulfonate sodium salt (DSS) [23]. The buffer used for the NMR experiments contained 90% H₂O/10% D₂O and 10 mM Bis-Tris, 15 mM NaCl at pH 7.2. For NMR spectroscopy, domain five of β 2GPI uniformly labeled with ¹⁵N was produced in *E.coli* by growth in M9 minimal media supplemented with ¹⁵N ammonium chloride. The ¹H-¹⁵N correlation spectrum of domain five was assigned previously [24]. Titrations of ¹⁵N-labeled domain five were performed with increasing concentrations of fondaparinux.

2.5. Docking of fondaparinux onto domain five of β 2GPI

Docking of fondaparinux onto domain five of β 2GPI (β 2GPI-DV) was performed with the program GOLD 5.1 [25]. The coordinates of β 2GPI-DV (PDB ID: 3OP8) and fondaparinux (PDB ID: 3EVJ) were taken from the Protein Data Bank, converted to a mol2 format and corrected to GOLD atom and bond types. All water molecules and bound sulfates were removed from the β 2GPI-DV structure and only chain A was retained for docking. The docking area was defined as a 14 Å radius centered at the C α atom of Lys 251. All rotatable bonds in fondaparinux were fixed. Docking was performed using GoldScore function with default settings to calculate 50 structures. Solutions were sorted based on protein-ligand hydrogen bond energy and the fifteen best structures were examined. The structure that corresponds the best to mutagenesis data and cardiolipin ELISA measurements (see Results) was selected. This structure was energy minimized using the CHARMM program [26]. The

sidechains within 6 Å from fondaparinux were treated as flexible during energy minimization.

2.6. Cardiolipin ELISA

Polystyrene 96 well plates (Costar) were coated with 50 µl per well of cardiolipin (Sigma) prepared at 200 µg/ml in absolute ethanol. Plates were blocked for 2 hours with 20 mM Tris, 100 mM NaCl buffer, pH 7.4 supplemented with 4% BSA. 2GPI (4 nM) with test reagents was preincubated with peroxidase-conjugated anti-2GPI antibodies (Cedarlane, 1:2500 dilution) in 20 mM Tris, 100 mM NaCl, 2mM CaCl₂ buffer, pH 7.4 for 1 hour at room temperature. Test reagents include heparin, enoxaparin, fondaparinux and A1-A1. Molar concentrations of heparin and enoxaparin were calculated assuming average molecular weights of 12000 Da and 4500 Da, respectively. Samples were added to wells (50 µl per well) and incubated for 1 hour at room temperature. Bound 2GPI/anti- 2GPI antibody complexes were detected using 2,2'-Azinobis [3-ethylbenzothiazoline-6-sulfonic acid]-diammonium salt (ABTS) substrate by measuring OD at 405 nm. The inhibition data was fitted to one-site models using the nonlinear least-squares Marquardt-Levenberg algorithm implemented in GNPLOT program. The fits of the raw data and the titration data points were then normalized to the maximum binding determined from the fit to facilitate comparison. We verified that heparin has very little effect on the binding of anti- 2GPI antibodies to 2GPI (Appendix A, Figure A.2).

2.7. Inhibition of the binding of β2GPI/anti-β2GPI antibody complexes to HUVEC by heparin, and fondaparinux

HUVEC were grown to confluence on 96-well microtiter plates first for 24 hours in EBM-2 media (Lonza) and then for 24 hours in EBM-2 media without heparin. Cells were washed with DMEM without serum and incubated with DMEM without serum for 2h at 37 °C. Test samples containing 5.4 µg/ml 2GPI and 10.8 µg/ml anti- 2GPI antibodies (Cedarlane) in the presence of various concentrations of either heparin, or fondaparinux, or anti- 2GPI antibodies alone were incubated for 1 hour at room temperature. Cells were put on ice, washed and incubated with the test samples, 50 µl per well, for 1 hour. Cells were washed and fixed with 0.1% glutaraldehyde for 10 min on ice. Binding of the anti- 2GPI antibodies was detected with HRP-conjugated secondary antibodies using TMB chromogenic reagent by measuring OD at 450 nm.

3. Results

3.1 Binding of fondaparinux to β2GPI-DV

2GPI binds heparin by its domain five [20]. Therefore, we used domain five of 2GPI (2GPI-DV) to detect the binding of fondaparinux and determine the binding affinity of the complex. 2GPI-DV was titrated with fondaparinux and changes in the protein tryptophan fluorescence were monitored and analyzed (Figure 1A). Titrations were performed in a buffer containing different concentrations of NaCl. We found that fondaparinux interacts with 2GPI-DV and that the binding is inversely dependent on the concentration of NaCl. In the presence of 100 mM NaCl, the measured K_d was 2 µM and stoichiometry of the complex was 1:1. Thermodynamic parameters of the binding and their dependence on salt concentration were further analyzed by isothermal titration calorimetry at 298K (Figure 1B and Table 1). Measured K_d values were 4.4 µM, 14.7 µM and 37.7 µM in the presence of 50 mM, 100 mM and 150 mM NaCl in the binding buffer, respectively, with one binding site for fondaparinux on 2GPI-DV. The K_d values measured by ITC, although slightly higher than that determined by monitoring of tryptophan fluorescence, show the same trend. The binding is in the low micromolar range, the binding affinity decreases with the increase of NaCl in the buffer and the stoichiometry of the complex is 1:1. To estimate the number of

acidic groups in fondaparinux involved in complex formation, we analyzed the salt-dependent contribution to the binding using the polyelectrolyte theory [27,28]. Multiple acidic groups in heparin are shielded by bound Na^+ ions. Upon binding to positively charged protein sidechains, sodium ions are displaced contributing to total free energy of binding. This salt-dependent component of the binding energy is purely entropic [28] and can be calculated from the equation: $\log K_d = \log K_{d_{\text{nonionic}}} + N^* \cdot \log[\text{Na}^+]$, where N is the number of acidic groups interacting with protein and f is the fraction of acidic groups shielded by Na^+ ions ($f = 0.8$ for heparin). Analysis of the slope and intercept of the linear fit of a salt plot (Figure 1C) resulted in 2.5 ionic contacts in the complex and -3.8 kcal/mol of salt-independent contribution to the binding energy. Therefore, the salt-independent part accounts for 52%, 58% and 63% of the total free energy of binding at 50 mM, 100 mM and 150 mM NaCl, respectively. The salt-independent component of the binding energy was further divided into entropic and enthalpic components (Figure 1D). Hydrogen bonds formed between fondaparinux and 2GPI-DV, and favorable van der Waals contacts account for enthalpic contribution to the binding energy. The loss of conformational degrees of freedom of both fondaparinux and 2GPI-DV upon complex formation results in the unfavorable entropic term in the salt-independent part of the binding energy (Figure 1D). In conclusion, analysis of the salt-dependent contribution to the binding indicates that at least three acidic groups of fondaparinux form hydrogen bonds with 2GPI-DV.

3.2. The binding site for fondaparinux on 2GPI-DV

We mapped the binding interface in the complex between 2GPI-DV and fondaparinux using solution NMR spectroscopy by monitoring perturbations of the resonances on the ^1H - ^{15}N correlation spectra of ^{15}N -labeled 2GPI-DV titrated with fondaparinux (Figure 2 A, B, C). Each peak on the NMR spectrum corresponds to an amide bond in 2GPI-DV and was previously assigned to a residue number in its primary sequence [24]. The residues of 2GPI-DV that either directly bind fondaparinux or are in close proximity to the binding site shift the most on the spectrum in response to increasing concentrations of fondaparinux (Figure 2 B, C). When mapped onto the structure of 2GPI-DV, the perturbed residues include residues from the flexible loop and Lys 251 pointing towards this loop. Interestingly, in the crystal structures of 2GPI, the backbone amide of Lys 251 forms a hydrogen bond with the sidechain of Glu 309 in the flexible loop (PDB ID: 1C1Z, 1QUB, 3OP8).

3.3. Lys 251 in 2GPI is critical for the binding of fondaparinux

We confirmed the importance of Lys 251 for fondaparinux binding by site directed mutagenesis combined with the protein fluorescence binding assay. We used two sets of mutations in 2GPI-DV and compared the effect of these mutations on the binding (Figure 3 A). In one of the mutants, Leu was substituted for Met 271. The second mutant combined Met271/Leu substitution with the mutation of two lysine residues, Lys 250 and Lys 251, to aspartates. Structures of the purified mutants were assessed by 1D proton NMR, confirming that the mutants are well structured (Appendix A, Figure A.1). Both lysine residues, Lys 250 and 251, are in a flexible region of 2GPI and are solvent exposed (Figure 3 A). Although we did not observe by NMR a shift of the backbone amide of Lys 250 in response to fondaparinux, it is possible that the interaction at the lysine sidechain does not transmit to conformational rearrangements at the backbone. Also, the double mutation will help to avoid possible reorientation of the Lys 250 sidechain and its contribution to the binding if only Lys 251 is mutated. Titrations were performed in a 10 mM bis-Tris, pH 7.0 buffer containing 100 mM NaCl. The Met 271 residue is part of the cluster of hydrophobic residues. It is far from the binding interface and, as expected, Met271/Leu mutation did not disrupt the binding of fondaparinux to 2GPI-DV. Titration data fitted to the quadratic binding equation resulted in a K_d value of 10 μM . While Met271/Leu mutation had no

effect on the binding of fondaparinux, the Lys250-251/Asp-Asp mutant completely lost the capability to bind fondaparinux, confirming the critical role of Lys 251 (Figure 3B).

3.4. Inhibition of the binding of β 2GPI/anti- β 2GPI antibody complexes to cardiolipin

The binding of β 2GPI/anti- β 2GPI antibody complexes to negatively charged phospholipids exposed on the surfaces of activated cells is one of the likely mechanisms leading to the pathology of antiphospholipid syndrome. A set of residues involved in the binding of β 2GPI to negatively charged phospholipids, Lys 284, Lys 286 and Lys 287, is also critical for the binding of heparin to β 2GPI [20]. We compared heparin, fondaparinux and a low-molecular weight heparin, enoxaparin, for the inhibition of the binding of β 2GPI/antibody complexes to the negatively charged phospholipid cardiolipin (Figure 4A). β 2GPI was incubated with anti- β 2GPI antibodies in the presence of test reagents and then applied to cardiolipin-coated plates. Heparin, enoxaparin and fondaparinux all inhibited the binding of β 2GPI/anti- β 2GPI antibody complexes to cardiolipin, but the inhibition was very weak. The concentrations of heparin, enoxaparin and fondaparinux at the half-maximal inhibition were 100 μ M, 230 μ M and 670 μ M, respectively. These concentrations are much higher than therapeutic doses of these drugs.

Previously, we demonstrated that a polypeptide molecule, A1-A1, effectively interfered with the binding of β 2GPI/anti- β 2GPI antibody complexes to cardiolipin [9]. A1-A1 consists of two identical ligand-binding domains A1 of ApoER2, connected together with a flexible linker. The two A1 domains in the A1-A1 polypeptide bind two β 2GPI molecules in the β 2GPI/anti- β 2GPI antibody complex, stabilizing it. A1-A1 inhibits the binding of β 2GPI/anti- β 2GPI antibody complexes to cardiolipin much more strongly than heparin. The half-maximal inhibition was achieved at 5 μ M of A1-A1 compared to 100 μ M of heparin (Figure 4A).

3.5. Effect of bound fondaparinux on the inhibition of the binding of β 2GPI/anti- β 2GPI antibody complexes to cardiolipin by heparin or A1

Previously, it was shown that Lys 284, 286 and 287 of β 2GPI are critical for the binding of heparin, and Lys 308 and 317 of β 2GPI are central for the binding of A1 [20,24]. A1 is the first ligand-binding domain of ApoER2. We investigated whether the binding site for fondaparinux on β 2GPI-DV overlaps with the lysine residues critical for interactions of β 2GPI-DV with heparin or A1. We incubated β 2GPI/anti- β 2GPI antibody complexes with 100 μ M fondaparinux. At this concentration, more than 87% of β 2GPI is in a complex with fondaparinux. We then evaluated the effect of bound fondaparinux on the inhibition of the binding of β 2GPI/anti- β 2GPI antibody complexes to cardiolipin by heparin and A1. When fondaparinux is bound to β 2GPI, heparin is less effective in inhibition of the binding of β 2GPI/anti- β 2GPI antibody complexes to cardiolipin (Figure 4B). In the presence of fondaparinux, the concentration of heparin at half-maximum inhibition increased from 100 μ M to 160 μ M, indicating that the binding site for heparin on β 2GPI partially overlaps the binding site for fondaparinux. The binding site for fondaparinux, which we mapped to a region in the vicinity of Lys 251 and 250, is far from lysines 284, 286 and 287, which are critical for the binding of heparin. The long heparin chain likely spans to the binding site used by fondaparinux. Bound fondaparinux makes heparin binding weaker but does not disrupt it completely.

Previously, we determined that A1 binds β 2GPI-DV with a low micromolar affinity, and the lysine residues 308 and 317 are critical for the binding [24]. In the structure of the β 2GPI-DV complex with A1, the binding site for A1 does not overlap with Lys 251, 250 [24]. Supporting this observation, the concentration of A1 at half-maximum inhibition of the binding of β 2GPI/anti- β 2GPI antibody complexes to cardiolipin remained about 70 μ M

regardless of the presence or absence of fondaparinux (Figure 4 C). This result strongly suggests that lysine residues 308 and 317 do not contribute to the binding of fondaparinux. Also, much lower inhibition efficiency of fondaparinux (670 μM) compared to A1 (70 μM) indicates that fondaparinux binds away from cardiolipin-binding sites on $\beta 2\text{GPI}$. Indeed, we know that affinities of fondaparinux and A1 for $\beta 2\text{GPI}$ are similar, and A1 binds $\beta 2\text{GPI}$ between the two phospholipid-binding sites efficiently precluding its binding to negatively charged surfaces. Moreover, high value of the half-maximal inhibition by fondaparinux (670 μM) indicates that the inhibition is likely a result of a non-specific binding of fondaparinux to lysine residues proximal to phospholipid-binding regions on $\beta 2\text{GPI}$.

3.6. Docking model of the complex between $\beta 2\text{GPI}$ -DV and fondaparinux

To build the structure of a complex of $\beta 2\text{GPI}$ -DV with fondaparinux, we used the docking program GOLD [25]. We calculated 50 docking structures and sorted them based on the external hydrogen bond score. The fifteen best structures were carefully examined to select a structure which is in agreement with our experimental observations. The following criteria were used to select the model of the complex that best fits all experimental observations: 1) involvement of Lys 251, 250 in the binding to the extent that the elimination of these interactions disrupts the binding completely, 2) no contribution from Lys 308 and Lys 317 in the loop region, 3) there are no steric clashes with the bound A1 molecule, 4) the binding site is not close to cardiolipin-binding regions on $\beta 2\text{GPI}$ (residues 284-287 and 313-316) and 5) at least three acidic groups of fondaparinux are engaged in the complex formation. Nine out of the fifteen models violated the experimental criteria. In the remaining models, fondaparinux was positioned between the lysine residues 246, 250, 251, 262 and 268. We selected the one with the best GOLD van der Waals score. The docking model of the complex between $\beta 2\text{GPI}$ -DV and fondaparinux which is in agreement with experimental data is shown in Figure 5 A,B,C. In the complex, fondaparinux binds to a band formed by the sidechains of lysine residues 246, 250, 251, 262 and 268. These sidechains, together with the sidechain of Gln 264, form hydrogen bonds with acidic groups on fondaparinux (Appendix A, Table A.1). In the complex, each of the five saccharides of fondaparinux forms at least one hydrogen bond with $\beta 2\text{GPI}$ -DV. Lys 251 interacts with saccharides 4 and 5, and Lys 250 binds saccharides 2 and 3 (saccharides in fondaparinux are numbered from 1 to 5 starting from the non-reducing end). Elimination of these interactions would decrease the binding. Moreover, mutation of Lys 250, 251 to aspartates would create a negative patch precluding the binding of highly negatively charged fondaparinux, as we observed in the binding assay for the Lys250-251/Asp-Asp mutant.

3.7. Fondaparinux at concentrations far exceeding therapeutic does not inhibit the binding of $\beta 2\text{GPI}$ /anti- $\beta 2\text{GPI}$ antibody complexes to HUVEC

The binding of $\beta 2\text{GPI}$ /anti- $\beta 2\text{GPI}$ antibody complexes to endothelial cells leads to cellular activation both in vitro and in vivo [29,30,31,32]. Patients with antiphospholipid syndrome have elevated plasma levels of markers for endothelial activation [33]. We treated HUVEC with the increasing concentrations of fondaparinux or heparin, and measured how their presence influenced the binding of $\beta 2\text{GPI}$ /anti- $\beta 2\text{GPI}$ antibody complexes to cells. Fondaparinux at concentrations up to 200 μM did not inhibit the binding of $\beta 2\text{GPI}$ /anti- $\beta 2\text{GPI}$ antibody complexes to cells (Figure 6 A, B). Only very high concentration (2.4 mg/ml) of heparin, which exceeds more than 100 fold its therapeutic dose, could inhibit the binding of $\beta 2\text{GPI}$ /anti- $\beta 2\text{GPI}$ antibody complexes to HUVEC, but even at this concentration the inhibition was not efficient.

4. Discussion

Antiphospholipid syndrome is a common thrombotic disorder with a poorly understood etiology. Thrombotic complications in APS are related to the presence of anti- β 2GPI antibodies. Treatments of thrombosis in APS are limited to general antithrombotic medications, but these drugs are not always effective [19]. Heparin, which is often used for prevention and treatment of thrombosis, can bind β 2GPI. It was suggested previously that heparin might act specifically on prothrombotic β 2GPI/antibody complexes [20]. Heparin interacts with domain V of β 2GPI at the site encompassing lysine residues 284, 286 and 287, and facilitates cleavage of β 2GPI by plasmin [20]. Because of unfavorable pharmacokinetics, heterogeneity and side effects associated with the use of heparin, LMWH and the synthetic pentasaccharide fondaparinux are often used instead of heparin. In this paper, we compared fondaparinux to heparin regarding their binding to β 2GPI. We also evaluated the efficiency of fondaparinux, enoxaparin and heparin in inhibition of the binding of β 2GPI/antibody complexes to negatively charged phospholipids and endothelial cells. Interaction of β 2GPI/antibody complexes with anionic phospholipids is believed to be critical for the pathology of APS and markers of endothelial activation are detected in APS patients [33].

We located the binding site for fondaparinux on domain five of β 2GPI and determined that at NaCl concentrations between 50 mM and 150 mM, fondaparinux binds β 2GPI-DV with a micromolar affinity forming a 1:1 complex. Here, we present a docking model of the complex between β 2GPI-DV and fondaparinux (Appendix B). In the complex, fondaparinux binds to a group of positively charged lysine sidechains, Lys 246, Lys 250, Lys 251, Lys 268, Lys 262, and to Gln 264. The docking model of the complex between β 2GPI-DV and fondaparinux is supported by multiple experimental observations. First, experimental NMR titration data identified residues on β 2GPI perturbed by the presence of fondaparinux, highlighting Lys 251 as the residue involved in complex formation. Using site-directed mutagenesis combined with the binding experiment, we confirmed the central role of Lys 251 in the complex. The Lys250-251/Asp-Asp mutant completely lost its ability to bind fondaparinux. In addition, fondaparinux does not interfere with the inhibition of the binding of β 2GPI/antibody complexes to cardiolipin by A1 and, therefore, does not overlap with the binding site for A1 known to be centered on Lys 308 and Lys 317 [24]. Moreover, fondaparinux only marginally perturbs the binding of β 2GPI/antibody complexes to cardiolipin, suggesting that fondaparinux binds away from the two regions on β 2GPI-DV known to be important for interactions with anionic phospholipids. These two regions include a sequence of hydrophobic residues 313-316 and a lysine-rich region from residue 281 to 288 [34,35,36,37]. A weak effect of fondaparinux on cardiolipin binding might be explained by non-specific association of fondaparinux with a cluster of lysine residues at the phospholipid binding site on β 2GPI.

In the complex, each of the five saccharides in fondaparinux is involved in at least one hydrogen bond with β 2GPI-DV. Dependence of the binding affinity on the concentration of NaCl in the buffer analyzed with the polyelectrolyte theory predicted that three Na^+ ions will be displaced from fondaparinux upon binding to β 2GPI-DV. In the complex, five sulfate and two carboxylate groups of fondaparinux form hydrogen bonds with β 2GPI-DV. It is not uncommon that a smaller number of acidic groups is predicted to interact with a protein than the number of bonds detected in atomic resolution structures of the protein complexes with DNA or polysaccharides [28].

The sidechains of the residues Lys 246, Lys 250, Lys 262, Lys 268 and Gln 264 bind fondaparinux in our model of the complex, but the resonances of their backbone amides shift very little in the NMR titration experiment. On the other hand, residues in the flexible loop

region are perturbed by bound fondaparinux even in the absence of direct binding. This contradiction can be explained by the following. In the NMR titration experiment we monitored changes at the backbone, there as fondaparinux interacts with amino groups at the end of long lysine sidechains. Sidechain interaction produces very little change in the backbone conformation, especially when backbone is constrained. Each of the fondaparinux-binding residues except for Lys 251, which is perturbed by bound fondaparinux, has neighboring hydrophobic residue forming a hydrophobic cluster. These are the residues Leu 247, Val 249, Ile 263 and Phe 267. The hydrophobic cluster together with Pro 248 rigidifies the backbone so that the interaction at lysine sidechain does not transmit to conformational rearrangement at the backbone. On the other hand, in the crystal structures of b2GPI (PDB ID: 1C1Z, 1QUB, 3OP8), the backbone amide of Lys 251 form hydrogen bond with the sidechain of Glu 309 in the flexible loop region. The backbone shift of Lys 251 that we observed by NMR easily translates to the backbone shift at Glu 309 and surrounding residues in the highly flexible loop, explaining the observed perturbations in the loop region in the absence of direct binding to fondaparinux.

Interestingly, the binding site for fondaparinux does not overlap with the residues, Lys 284, Lys 286, Lys 287, known to be critical for the binding of heparin [20]. Heparin binds to one of two regions on 2GPI required for the interaction with negatively charged phospholipids [34,35,36,37]. We have demonstrated that bound fondaparinux only partially perturbed the ability of heparin to interfere with the binding of 2GPI to cardiolipin. This result suggests that long heparin chain likely binds to two sites on 2GPI. The major high affinity heparin-binding site is around the cardiolipin-interacting region of 2GPI including residues Lys 286, Lys 287 and the other low affinity site, which is preferred by fondaparinux, is around Lys 251. Our model of the 2GPI complex with fondaparinux explains why the binding site for fondaparinux is not the major binding site for heparin. The most common disaccharide in heparin is composed of a 2-O-sulfated iduronic acid and 6-O-sulfated, N-sulfated glucosamine, IdoA(2S)-GlcNS(6S). If this disaccharide is substituted for saccharides 2 and 3 of fondaparinux, this new pentasaccharide will lose two hydrogen bonds with Lys 250. In addition, the new pentasaccharide will have a sulfate group in a saccharide 2, which is not present in fondaparinux. This sulfate group will be too close to the aliphatic portion of the Lys 250 sidechain, forcing pentasaccharide to shift and further disturbing hydrogen bonds between Lys 246 and saccharides 1, 2 and 3.

The binding of 2GPI and 2GPI/antibody complexes to negatively charged surfaces plays an important role in antiphospholipid syndrome. The binding of circular 2GPI circulating in the blood to negative phospholipids leads to a conformational change in 2GPI, exposing additional epitopes for pathological anti- 2GPI antibodies [4]. Anti- 2GPI antibodies strengthen the attachment 2GPI to negatively charged surfaces. Also, the inhibition of the assembly of ordered structures of annexin V on anionic phospholipids on activated cells is the likely prothrombotic mechanism of the 2GPI/antibody complexes [38]. Our data strongly suggests that neither heparin nor fondaparinux efficiently interfere with the binding of 2GPI/antibody complexes to negatively charged phospholipids. Although heparin and fondaparinux bind 2GPI at therapeutically relevant doses, concentrations of heparin and fondaparinux at half-maximal inhibition of the 2GPI/antibody complexes to cardiolipin are much higher than their therapeutic doses [39]. Also, neither fondaparinux nor heparin prevented the binding of the 2GPI/antibody complexes to endothelial cells even at concentrations exceeding their therapeutic doses more than 100 fold. Because fondaparinux binds 2GPI with much lower affinity than antithrombin III, which concentration in plasma is 2.6 μ M, fondaparinux is bound predominantly to antithrombin III at therapeutic doses [40].

Despite the absence of a direct therapeutic effect of fondaparinux on α_2 GPI, the binding site for fondaparinux identified in this study is a potential 'hot spot' for other ligands of α_2 GPI. It is well established that protein-ligand interactions are localized to a limited number of preferred or 'hot' spots on a protein surface [41,42]. The current understanding of molecular mechanisms of antiphospholipid syndrome is incomplete and very little is known about the binding sites for the cell-surface receptors on α_2 GPI. The binding site for fondaparinux on α_2 GPI that we identified might point to a binding site for a α_2 GPI receptor.

5. Conclusion

We present the docking model of the structure of α_2 GPI with fondaparinux and compared fondaparinux to heparin regarding its ability to bind α_2 GPI and inhibit the binding of α_2 GPI/antibody complexes to negatively charged phospholipids and endothelial cells. Interaction of α_2 GPI/antibody complexes with anionic phospholipids and endothelial cells is believed to be critical for the pathology of APS. We determined that the binding site for fondaparinux on α_2 GPI does not include lysine residues known to be critical for the binding of heparin. It is likely that long heparin chain spans two sites, high- and low-affinity sites, on α_2 GPI. The docking model of the complex between α_2 GPI and fondaparinux presented here is in agreement with multiple experimental observations. We found that fondaparinux binds α_2 GPI at therapeutically relevant concentrations. Although heparin and fondaparinux bind α_2 GPI at therapeutically relevant doses, they are not efficient in interfering with the binding of α_2 GPI/antibody complexes to negatively charged phospholipids and endothelial cells. Our results suggest that these drugs do not act on pathological properties of α_2 GPI/antibody complexes emphasizing the need for a new treatment specific for α_2 GPI-related thrombosis in APS.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Highlights

- Heparin and fondaparinux are used for prevention and treatment of thrombosis in APS
- Fondaparinux forms 1:1 complex with 2GPI
- The K_d value of the complex is in the low micromolar range
- Fondaparinux and heparin interact with different sites on 2GPI
- Fondaparinux does not prevent the binding of 2GPI to cardiolipin and EC

Figure 1.

Binding of fondaparinux to domain five of 2GPI. A) Changes of tryptophan fluorescence of 2GPI-DV titrated with fondaparinux in the presence of 15 mM NaCl (triangles) and 100 mM NaCl (circles). B) ITC titrations of 2GPI-DV (50 μ M) with fondaparinux (750 μ M) in 25 mM cacodylate buffer, pH 7.2 in the presence of 50 mM NaCl, 100 mM NaCl and 150 mM NaCl. Titrations were performed at 298K. The upper half of the figure shows ITC data after correction for heat of dilution. The lower half of the figure shows the binding isotherm fitted to a single site binding model. The K_d values are 4.4 μ M (50 mM NaCl), 14.7 μ M (100 mM NaCl) and 37.4 μ M (150 mM NaCl). C) The salt dependence of the dissociation constants of the 2GPI-DV complex with fondaparinux measured by ITC at 50 mM, 100 mM and 150 mM NaCl. D) Comparison of the binding thermodynamics of 2GPI-DV complexes with fondaparinux at 298K in the presence of 50 mM, 100 mM and 150 mM NaCl in the binding buffer. The free energies of binding, ΔG , were calculated from the K_a values. ΔG is shown as open bars, ΔH black, $T\Delta S_{\text{non}}$ gray and $T\Delta S_{\text{ion}}$ is hatched.

Figure 2. Mapping the contact site for fondaparinux onto 2GPI-DV

A) Superposition of the ^{15}N -HSQC spectra of 2GPI-DV in isolation (black) and in the presence of 1:0.3 (green) and 1: 1 (red) molar ratio of 2GPI-DV to fondaparinux. B) Combined, ^1H and ^{15}N , chemical shift perturbations of 2GPI-DV in the presence of fondaparinux. Chemical shift difference between the spectra of 2GPI-DV in the absence and in the presence of 1:1 molar ratio of fondaparinux was calculated as $=\sqrt{H^2 + (0.15 \cdot N)^2}$. The last bar corresponds to tryptophan sidechain. C) Chemical shift perturbations mapped onto the structure of 2GPI-DV. Residues that are shifted more than one standard deviation above average are colored purple and residues that are missing from the spectra are grey.

Figure 3.

Lys 251 is critical for the binding of fondaparinux to 2GPI-DV. A) Mutated residues, Met 271, Lys 250 and Lys 251, in the context of the 3D structure of 2GPI-DV. Met 271 (blue) is part of the cluster of hydrophobic residues (grey) in 2GPI-DV. The binding of fondaparinux is monitored by the changes in the intrinsic fluorescence of Trp 316 (cyan). B) Changes of tryptophan fluorescence of the Met271/Leu (circles) and Met271/Leu, Lys250-251/Asp-Asp (triangles) mutants of 2GPI-DV titrated with fondaparinux in the presence of 100 mM NaCl. Titration data for the Met271/Leu mutant fitted to a quadratic equation resulted in K_d value of 10 μM .

Figure 4.

Inhibition of the binding of 2GPI/anti-2GPI antibody complexes to cardiolipin. A) The binding of 2GPI/anti-2GPI antibody complexes to cardiolipin in the presence of increasing concentrations of heparin (diamonds), enoxaparin (triangles), fondaparinux (circles) and A1-A1 (inverted triangles). The half-maximum inhibition of the binding was achieved by 100 μ M heparin, 230 μ M enoxaparin, 670 μ M fondaparinux and 5 μ M of A1-A1. B) Inhibition of the binding of 2GPI/anti-2GPI antibody complexes to cardiolipin by heparin in the absence (triangles) and presence (circles) of saturating concentrations of fondaparinux. The half-maximum inhibition of the binding was achieved by 100 μ M heparin in the absence of fondaparinux and 160 μ M heparin in the presence of saturating concentrations of fondaparinux. C) Inhibition of the binding of 2GPI/anti-2GPI antibody complexes to cardiolipin by A1 in the absence (triangles) and presence (circles) of the saturating concentrations of fondaparinux. At half-maximum inhibition, the concentration of A1 is about 70 μ M regardless of the presence or absence of fondaparinux. Fitting to a one-site inhibition model was performed on raw data. To facilitate comparison, the measured OD_{405} values and the inhibition curves were normalized to the maximum binding obtained from the fit. The errors of measured binding ($n=3$) are indicated.

Figure 5.

Structure of the complex between 2GPI-DV and fondaparinux. A) 2GPI-DV is green. The lysine residues Lys 251 critical for fondaparinux binding and Lys 284, 286 and 287 critical for the binding of heparin are shown in stick representation and labeled. Fondaparinux is shown as a stick model. B) Surface representation of 2GPI-DV colored by electrostatic surface potential, with bound fondaparinux shown as a stick model. The lysine residues forming polar contacts with acidic groups of fondaparinux are labeled. C) Stereo view of the complex between 2GPI-DV, fondaparinux and A1. 2GPI-DV in the complex with the ligand-binding domain from the lipoprotein receptor (PDB ID: 2KRI) was superimposed with 2GPI-DV in the 2GPI-DV complex with fondaparinux. 2GPI-DV is purple, A1 is blue and the structure of fondaparinux is shown as a stick model. Two regions on 2GPI-DV critical for the binding to anionic phospholipids (lysine residues 284, 286 and 287 and hydrophobic residues 313-316) are colored green.

Figure 6. Inhibition of the binding of the 2GPI/anti-2GPI antibody complexes to HUVEC by A) fondaparinux and B) heparin. All points were measured in triplicate. The last bar in the histograms (black) is the bound anti-2GPI antibody in the absence of added 2GPI. Bars represent the average values of mean and standard deviation of four independent experiments (fondaparinux) and three independent experiments (heparin).

Table 1

Thermodynamics of fondaparinux binding to 2GPI-DV.

NaCl (mM)	N	K _a (M ⁻¹)	H (cal/mol)	T S (cal/mol) ^a	G (cal/mol)
50	0.93 ± 0.01	225800 ± 18630	-10330 ± 188	-3010	-7320
100	0.96 ± 0.05	68120 ± 9343	-7067 ± 505	-474	-6593
150	1.08 ± 0.12	26540 ± 5565	-4108 ± 637	1925	-6033

^aT=298K