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Exploration of the Antiplatelet Activity Profile of Betulinic Acid on Human Platelets

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Supporting Information

ABSTRACT: Betulinic acid, a natural pentacyclic triterpene acid, presents a diverse mode of biological actions including antiretroviral, antibacterial, antimalarial, and anti-inflammatory activities. The potency of betulinic acid as an inhibitor of human platelet activation was evaluated, and its antiplatelet profile against in vitro platelet aggregation, induced by several platelet agonists (adenosine diphosphate, thrombin receptor activator peptide-14, and arachidonic acid), was explored. Flow cytometric analysis was performed to examine the effect of betulinic acid on P-selectin membrane expression and PAC-1 binding to activated platelets. Betulinic acid potently inhibits platelet aggregation and also reduced PAC-1 binding and the membrane expression of P-selectin. Principal component analysis was used to screen, on the chemical property space, for potential common pharmacophores of betulinic acid with approved antithrombotic drugs. A common pharmacophore was defined between the NMR-derived structure of betulinic acid and prostacyclin agonists (PGI2), and the importance of its carboxylate group in its antiplatelet activity was determined. The present results indicate that betulinic acid has potential use as an antithrombotic compound and suggest that the mechanism underlying the antiplatelet effects of betulinic acid is similar to that of the PGI2 receptor agonists, a hypothesis that deserves further investigation.

KEYWORDS: *betulinic acid, platelet aggregation, ADP, antithrombotics*

INTRODUCTION

Thromobogenesis is a multicomponent and complex pathophysiological process that requires both humoral and cellular factors.[1] Platelets play a crucial role in arterial thrombosis. Platelet activation leads to shape change, secretion of granular contents and release of arachidonic acid, adhesion to the site of injury, and aggregation.[2]

To combat a complex pathophysiological process such as thrombogenesis, multitargeted drugs should be developed. Natural products present an important source of chemicals with such properties,[3−7] with approximately half of drugs currently used in the clinic having derived from natural products.[8] Natural products have evolved through natural selection to interact with multiple targets and to modulate multiple signal transduction pathways. Furthermore, natural products frequently resemble biosynthetic intermediates or endogenous metabolites and, thus, can favorably utilize native active transport mechanisms. Thus, natural products present an important source for identification of multitarget compounds.

A number of studies have suggested that certain bioactive chemicals present in plants may protect against thrombosis.[9−11] One such group of compounds is pentacyclic triterpenes from the lupane, oleanane, and ursane groups. Pharmacological relevance has increased during the past two decades, demonstrating multitarget properties combined with low toxicity.[12] One of the most promising multifunctional compounds that targets multiple steps in signal transduction pathways is betulinic acid (Scheme 1), which can be found in the bark of several species of plants and principally the white birch and ber trees.[13−15] Specifically, it has demonstrated a broad range of pharmacological actions including antimalarial properties,[16] topoisomerase inhibitory activity,[17] antitumor and anticancer properties,[18−20] and anti-inflammatory and antiretroviral activities.[21,22] To the best of our knowledge, the effect of betulinic acid as an inhibitor of human platelet activation has yet to be explored. This study aims to analyze and dissect the antiplatelet effect of betulinic acid in addition to studying the potential mechanisms involved in this action.

MATERIALS AND METHODS

Chemicals. Betulinic acid and betulin were purchased from Sigma (Steinheim, Germany). DMSO- d_6 (99.8%) was purchased from Deutero (Kastellaun, Germany). Adenosine diphosphate (ADP) was purchased from Chrono-Log Corp. (Havertown, PA, USA). Arachidonic acid (AA) and thrombin receptor activator peptide-14 (TRAP) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Fluorescently labeled monoclonal antibodies, PAC-1-FITC, anti-

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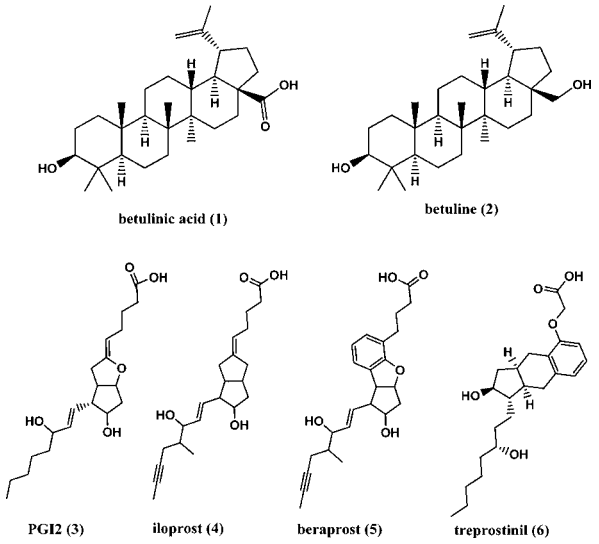
Scheme 1. 2D Structures of Betulinic Acid and Betulin[a]



[a]The 2D structure of prostacyclin and its synthetic analogues (iloprost, beraprost, treprostinil) show common features: the C1-COOH, the α-chain, the C11-OH, the C15-OH, and the ω-chain.

CD62P-PE, and anti-CD61-PerCP, were obtained from Beckton Dickinson (San Jose, CA, USA).

NMR Experiments and Modeling of Betulinic Acid and Betulin. NMR experiments were performed at 298 K on a Bruker AV-500 spectrometer equipped with a TXI cryoprobe (Bruker BioSpin, Rheinstetten, Germany). Samples were dissolved in 0.5 mL of DMSO-d_6 (final concentration = 5 mM for both betulinic acid and betulin) and transferred to 5 mm NMR tubes. The NMR system was controlled by the software TopSpin 2.1. 2D ^{1}H−^{1}H NOESY spectra were acquired using a spectral width of 6502 Hz, acquisition time of 0.079 s in F_2 and 0.020 s in F_1, relaxation delay of 2 s, and mixing time of 1000 ms (d8 = 1 s). Assignment was determined on the basis of 2D ^{1}H−^{13}C HSQC and HMBC spectra. Betulinic acid and betulin were modeled on the basis of NMR-derived distance restraints (NOEs) and available X-ray data.[23]

Principal Component Analysis (PCA). A total of 18 antithrombotic drugs (Figure 3) were selected for analysis. This relatively small data set allows for identification of individual compounds in the resulting chemical space plot. A set of 8 physicochemical and stereochemical properties for all 18 compounds was then calculated using chemaxon cheminformatic tools:[24] molecular weight (MW), nitrogens (N), oxygens (O), hydrogen bond donors (HBD), hydrogen bond acceptors (HBA), topological polar surface area (tPSA), stereogenic centers (nStereo), and solvent accessible/water accessible surface area. To provide a visual representation of the position of each compound in chemical space, we then carried out PCA by considering the Matlab Statistical Toolbox to reduce the 8-dimensional vector corresponding to each compound to a 2-dimensional vector, with minimal loss of information. PCA can be defined as the orthogonal projection of the original data onto a lower dimensional space, called the principal subspace, such that the variance of the projected data is maximized along its first axes.[25] This technique can be understood as a rotation of the axes of the original variable coordinate system to new orthogonal axes to make the new axes coincide with the directions of maximum variation of the original variables. Indeed, PCA also allows removal of the correlations between these features in an optimum fashion. Before implementation of the PCA, it is necessary to standardize the original data by subtracting the mean from each feature and dividing by its respective standard deviation. This step is necessary, because the features present different scales, which yields heterogeneous variances. The new transformed data features present zero mean and unit variance. We have implemented the standardization procedure by considering the Matlab software. In practice, PCA consists initially of finding the eigenvalues and eigenvectors of the sample covariance matrix, obtained from the attribute matrix; that is, a matrix in which the rows represent observations (each antithrombotic drugs) and the columns, each of the eight attributes. Subsequently, the eigenvectors are sorted in decreasing order according to their eigenvalues. The multiplication of the original data by the two main eigenvectors gives the data projection. All PCAs were performed using Matlab software. Hierarchical clustering has been also performed to identify the clusters of the antithrombotic drugs (see the Supporting Information).

Platelet Aggregation in Platelet-Rich Plasma (PRP). Platelet aggregation studies in PRP prepared from peripheral venous blood of apparently healthy volunteers were performed as we have previously described.[26] Briefly, the platelet count of PRP was adjusted to a final platelet concentration of 2.5×10^8/mL with homologous platelet-poor plasma (PPP). Platelet aggregation in the presence of ADP (10 μM), AA (0.5 mM), and TRAP (10 μM) was measured in aliquots of 0.5 mL of PRP, in a Chronolog Lumi-Aggregometer (model 560-Ca) at 37 °C, with continuous stirring at 1200 rpm. The maximal aggregation, achieved within 3 min after the addition of each agonist, was determined and expressed as a percentage of 100% light transmission calibrated for each specimen (maximal percentage of aggregation). Betulinic acid and betulin obtained as white powder were dissolved in DMSO. The final DMSO concentration in PRP did not exceeded 1% (by volume), a concentration that does not influence platelet activation.[27] The inhibitory efficacy of betulinic acid was expressed as IC_{50} values (concentration that induces 50% inhibition of platelet aggregation). All aggregation studies were conducted within 3 h of blood draw.

P-Selectin Membrane Expression and PAC-1 Binding. The surface expression of CD62P (P-selectin) and the PAC-1 binding to activated platelets were studied by flow cytometry in a FACSCalibur flow cytometer (Becton-Dickinson) using a slight modification of a technique previously described.[28] Briefly, platelets were incubated in the presence or in the absence of 440 μM betulinic acid or 300 μM betulin with ADP, AA, or TRAP (50 mM final concentration for each agonist) for 5 min at 37 °C. Platelets were then incubated with PAC-1-FITC and anti-CD62P-PE for 20 min in the dark at room temperature, diluted (1:5, v/v) with 10 mM PBS, pH 7.4, and immediately analyzed by flow cytometry (FACsCalibur, Becton-Dickinson) as previously described.[29] Platelets were gated according to staining for the platelet-specific antigen CD61. The gated events were further analyzed in histograms for FL-1 for PAC-1 and FL-2 for the detection of P-selectin, respectively. Analyses included the percentage of positive events facilitated by the evaluation of mean fluorescence intensity (MFI).

RESULTS AND DISCUSSION

The effect of betulinic acid and betulin on platelet aggregation in vitro was studied in human PRP activated by ADP, TRAP, and AA. As shown in Table 1, betulinic acid significantly

Table 1. Effect of Betulinic Acid (1) and Betulin (2) on Platelet Aggregation Induced by ADP, AA, and TRAP

		inhibition[a] (%)		
natural product	concn (μM)	ADP	AA	TRAP
betulinic acid (1)	440	32 ± 3.5	86 ± 11	80 ± 9
	220	19 ± 4.2	48 ± 7.4	60 ± 2.4
	176	8 ± 5	39 ± 2	45 ± 7
	88	5 ± 3	31 ± 2.5	38 ± 9.5
	63	0	22 ± 5	27 ± 5
	44	0	14 ± 2	16 ± 4
betulin (2)	300	0	7 ± 2	13 ± 3

[a]Data represent the mean ± SD values from at least three experiments. ADP, adenosine diphosphate; TRAP, thrombin receptor activator peptide-14; AA, arachidonic acid.

inhibited platelet aggregation induced by all agonists in a dose-dependent manner, the maximum inhibition being observed at a concentration of 440 μM (higher concentrations could not feasibly be tested due to solubility problems). Moreover, betulinic acid is more efficient in inhibiting platelet aggregation induced by AA and TRAP than ADP, with significantly higher percent inhibition values (Table 1) and lower IC_{50} values (210, 187, and 102 μM for ADP, AA, and TRAP, respectively). Typical aggregation curves illustrating the dose-dependent inhibitory effect of betulinic acid are presented in Figure 1. In

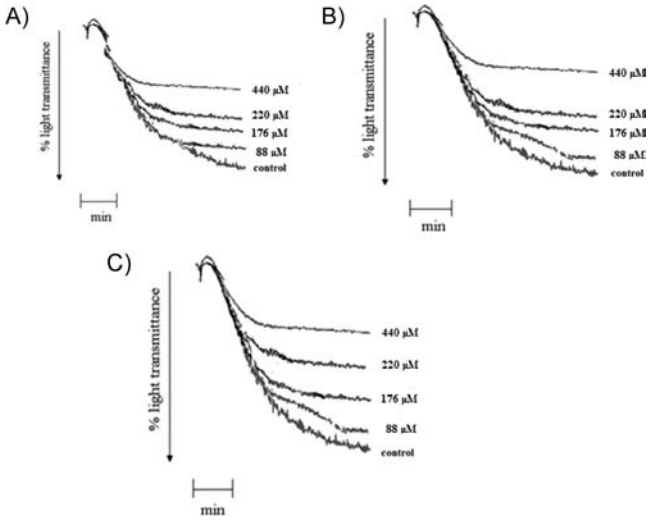


Figure 1. Dose–response curves for betulinic acid demonstrating the inhibition of platelet aggregation induced by ADP (A), arachidonic acid (AA) (B), and TRAP (C).

contrast to betulinic acid, betulin even at a high concentration (300 μM) similar to the highest concentration of betulinic acid used in the present study did not affect platelet aggregation by ADP, whereas only a marginal inhibition was observed in platelet aggregation induced by AA and TRAP. It should be noted that we could not use a concentration higher than 300 μM for betulin due to its much lower solubility in DMSO in comparison to betulinic acid.

The above results prompted us to further investigate the inhibitory effect of betulinic acid on platelet activation by studying the conformational change of the integrin receptor αIIb/β3 (PAC-1 binding) and the membrane expression of P-selectin. PAC-1 is a monoclonal antibody that binds to the activated form of the integrin receptor αIIb/β3.[30] The activation of this integrin leads to its conformational change and the recognition of various ligands, primarily fibrinogen, resulting in platelet aggregation and further activation through αIIb/β3-mediated outside-in signaling.[26] P-selectin is a major platelet α-granule protein that is highly expressed on the platelet surface during activation and plays a significant role in platelet–leukocyte and platelet–endothelial cell interactions.[31] As shown in Table 2, betulinic acid at 440 μM significantly inhibits PAC-1 binding and P-selectin expression induced by all agonists, maximal inhibition being observed when TRAP was used as agonist. By contrast, betulin failed to inhibit PAC-1 binding and P-selectin expression induced by all agonists (Table 2). Representative histograms illustrating the effect of betulinic acid and betulin on PAC-1 binding and P-selectin expression induced by TRAP are shown in Figure 2. The above inhibitory effects of betulinic acid, which are more potent when AA or TRAP is used as agonist compared with ADP, are in accordance with its inhibitory effects on platelet aggregation.

Having defined the potency of betulinic acid in inhibiting platelet activation, induced by three different agonists, we next aimed to define pharmacophores responsible for this activity. To investigate a potential overlap in the coverage of biologically relevant chemical space between betulinic acid and approved antithrombotic drugs, maps of the chemical space were produced from property spaces and visualized by PCA. A small database of 18 approved antithrombotic drugs (Figure 3) was constructed and grouped in five families according to their mode of action: cyclooxygenase-1 (COX-1) inhibitors, ADP receptor antagonists, prostacyclin (PGI2) IP receptor agonists, thromboxane receptor antagonists, and phosphodiesterase inhibitors. The property spaces describe eight calculated structural and physicochemical parameters such as size, polarizability, polarity, flexibility, and hydrogen bond capacity. PCA was utilized to replot the data in a two-dimensional format representing 84.1% of the original information in the full eight-dimensional data set (Figure 3). The two unitless orthogonal axes represent linear combinations of the original eight parameters.

Notably, PGI2 receptor agonists cluster largely in one region of the plot, and betulinic acid belongs also in this cluster (this is in accordance with a hierarchical clustering approach considering either the single or the Ward linkage function (see the Supporting Information, Figure S6)). In contrast, the target-specific ADP receptor antagonists, COX-1 inhibitors, thromboxane TP receptor antagonists, and phosphodiesterase inhibitors cover a different part of the chemical space. Analysis of component loadings indicates that, in general, antithrombotic drugs feature higher polar surface area compared to betulinic acid. From the studied drugs betulinic acid is closest, in the defined chemical space, to iloprost, which is an analogue of PGI2. The recorded similarity of betulinic acid to PGI2 and its analogues, on the chemical property space, by use of physicochemical descriptor metrics, prompted us to investigate the potential sampling of common pharmacophores.

Table 2. Effect of Betulinic Acid (1) and Betulin (2) on P-Selectin Expression and PAC-1 Binding

	inhibition[a] (%)					
	PAC-1 binding			P-selectin expression		
natural product	ADP	AA	TRAP	ADP	AA	TRAP
betulinic acid (**1**)	30.5 ± 2.4	51.0 ± 4.5	75.9 ± 4	22.6 ± 2.3	57.0 ± 2.2	93.9 ± 2.6
betulin (**2**)	3.4 ± 0.3	0.5 ± 1.4	4.9 ± 5.2	6.3 ± 0.4	11.0 ± 2.4	8.8 ± 3.9

[a]Values represent the inhibitory effect of each natural product at a concentration of 440 or 300 μM, respectively, on P-selectin (CD62P-PE) membrane expression and PAC-1 (PAC-1-FITC) binding induced by ADP, AA, and TRAP. Data represent the mean ± SD values from at least three experiments.

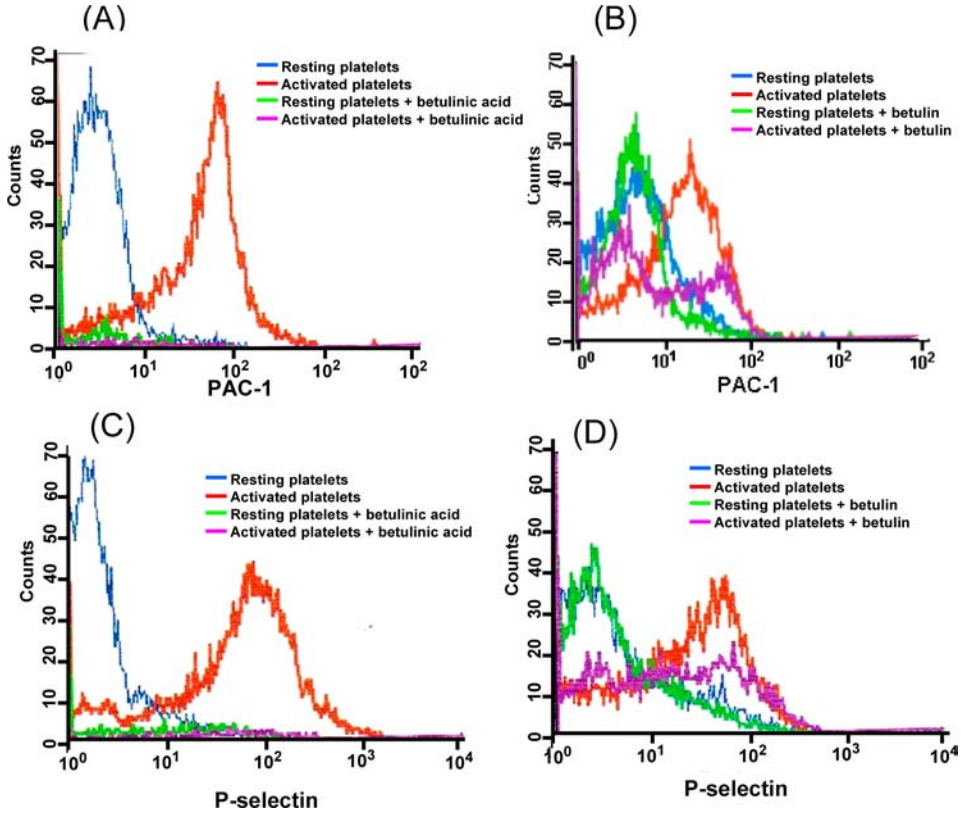


Figure 2. Representative histograms, obtained by flow cytometry analysis, illustrating the effect of betulinic acid (A, C) and betulin (B, D) on PAC-1-FITC binding and CD62P-PE membrane expression on activated with TRAP platelets, respectively.

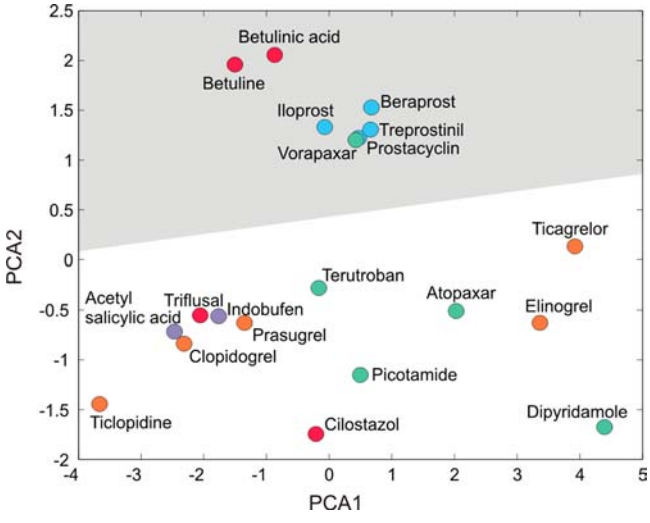


Figure 3. Principal component analysis (PCA) comparison of five families of 18 approved antithrombotic drugs representative of five mechanistic groups: adenosine diphosphate receptor inhibitors (orange); prostanoid prostacyclin receptor agonists (blue); COX inhibitors (purple); thromboxane inhibitors (green); and phosphodiesterase inhibitors (red). The first two principal components account for 84.1% of the original information. Clusters of antithrombotic drugs were identified by considering the hierarchical clustering approach (single-linkage method).[25] The four prostacyclin receptor agonists cluster largely in one region of the plot (gray area).

PGI2 is a potent endogenous vasodilator and inhibitor of platelet aggregation. Both actions are mediated by the IP receptor, which is a G-protein coupled receptor.[32] The PGI2 synthetic analogues beraprost, iloprost, and treprostinil (Scheme 1) have been successfully used in the clinical treatment of pulmonary arterial hypertension.[27] The main structural features of PGI2 analogues illustrate common features such as a carboxylate group at C1, the α-chain; a hydroxyl group at C11, the ω-chain; and the C15-OH (Scheme 1). Previously, a common pharmacophore was constructed for human IP receptor agonists.[33] The pharmacophore deduced from this study indicated the existence of three main structural characteristics: a carboxylate group, which is an essential feature for all agonists; a hydrogen bond accepting and/or donating group located at a distance of 8−11 Å from the carboxylate group; and a spacer among these groups formed by a relative and extended lipophilic area composed by aromatic or aliphatic side chains. This pharmacophore emphasized the 3D orientation of chemical functions necessary to binding to IP receptor and to express their inhibitor activity in platelet aggregation.[33] The importance of the conserved carboxylate group for all IP receptor ligands led to the assumption of a hydrogen bond between this carboxylate group and Arg279.[33]

Betulinic acid, similar to PGI2, is composed by the following chemotypes: a hydroxyl group at C-3, a carboxylate group at C-28, an alkene group at C-20, and a pentacyclic carbon skeleton. To determine whether betulinic acid chemotypes fold in the pharmacophore model of IP receptor agonists, we determined its 3D structure. The 3D architecture of betulinic acid was built on the basis of the recently determined X-ray structure of 3β-hydroxylup-20(29)-en-28-yl 1*H*-imidazole-1-carboxylate[23] and 2D ^{1}H−^{1}H NOESY NMR data (Supporting Information, Table S4). As can be seen in Figure 4, in the NMR-derived architecture, betulinic acid is composed by a carboxylate group that is located at a distance of 11.2 Å from the hydroxyl group, and these two chemotypes are connected by a planar

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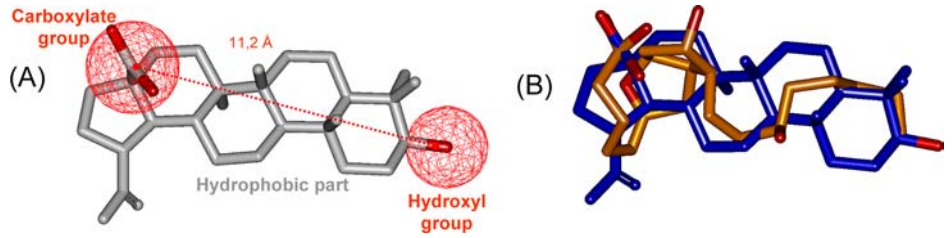

Figure 4. (A) NMR-derived 3D structure of betulinic acid. The two chemotypes of betulinic acid, the carboxylate group and the hydroxyl group, are highlighted, and their van der Waals surfaces are colored in red. (B) Superposition of the NMR-derived structure of betulinic acid to the synthetic PGI2 analogue iloprost. Iloprost is shown in its bound form to the hIP receptor as was previously determined.[34] Betulinic acid is colored in blue and iloprost in orange.

hydrophobic spacer composed by a tetracyclic ring system. Interestingly, these chemical groups of betulinic acid show spatial rearrangement similar to that of the pharmacophore of IP receptor agonists, suggesting that it could also participate in similar ligand binding interaction modes. Thus, it could be suggested that due to this similarity betulinic acid could exert its effects at least partially on the IP receptor. Our results showing that betulinic acid is more efficient in inhibiting platelet aggregation induced by AA and TRAP than ADP are in accordance with the above suggestion. To further support the above suggestion, we examined the agonist binding features of the ligand binding pocket of the human IP receptor. The agonist binding pocket of hIP became evident after the construction of a model of iloprost bound to the homology modeled hIP receptor.[34] Iloprost is a stable high-affinity hIP agonist that substitutes a secondary cyclopentane ring in place of the PGI_2 oxolane ring and carries an additional C16-methyl group and a ω-chain triple bond (Scheme 1). From the iloprost–hIP receptor 3D model the carboxylate group of iloprost was identified as a crucial site for its activity due to its participation in an ionic interaction with the highly conserved Arg279. Mutation of this residue in hIP resulted in a significant decrease in agonist binding affinity.[34]

Superposition of our NMR-derived structure of betulinic acid and the previously determined structure of iloprost, in its bound form to the hIP receptor,[34] emphasized a high overall structure similarity, suggesting that betulinic acid could be similarly accommodated in the same hIP binding pocket (Figure 4B). Interestingly, this highlighted structural similarity can be fruitfully linked to functional homology on the basis that, analogously to the platelet aggregation profile we found for betulinic acid, iloprost has been reported to inhibit platelet aggregation,[35−38] PAC-1 binding, and P-selectin expression.[38−40] Betulinic acid could therefore accommodate successfully the same ligand binding pocket in hIP as iloprost. A direct ionic interaction between the C17-COOH of betulinic acid and R279 could be formed as also extensive hydrophobic interactions of the pentacyclic carbon skeleton of betulinic acid to the receptor similarly to iloprost. On the basis of these observations and to probe the potential importance of the carboxylate group of betulinic acid in its activity, we explored the antiplatelet profile of betulin (Scheme 1). Betulin lacks a carboxyl group and, according to our aforementioned hypothesis, should present reduced antiplatelet activity in comparison to betulinic acid. Indeed, betulin had no effect on ADP, AA, or TRAP and induced platelet aggregation, even at highest concentrations. Moreover, betulin was not effective in inhibiting PAC-1 binding and P-selectin membrane expression.

In view of the development of multitargeted ligands as effective antithrombotic drugs, betulinic acid could be a good lead molecule. From our study a potential pharmacophore overlay between betulinic acid and PGI2 analogues was identified, suggesting a potential common function among these molecules. Through this similarity the importance of the carboxylate group of betulinic acid was suggested, and it was experimentally validated. This identified similarity between PGI2 and betulinic acid is of importance, because PGI2, besides being an effective antiplatelet inhibitor, has been implicated, similarly to betulinic acid, in inhibiting pathways involved in the development of cancer.[41−43] Although the detailed mechanism for the antiplatelet activity of betulinic acid remains to be clarified, the present results suggest that betulinic acid could be a useful antithrombotic agent.

■ ASSOCIATED CONTENT

Supporting Information

NMR (2D spectra, assignment, NOE correlations), approved antithrombotic drugs used in the study, details on the hierarchical clustering approach, and resulting dendrogramms. This material is available free of charge via the Internet at http://pubs.acs.org.

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Author Contributions

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Notes

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