European Journal of Medicinal Chemistry 94 (2015) 113–122

Contents lists available at ScienceDirect

European Journal of Medicinal Chemistry

journal homepage: http://www.elsevier.com/locate/ejmech

Original article

New platelet aggregation inhibitors based on pyridazinone moiety

Tamara Costas [a, b, 1], María Carmen Costas-Lago [a, b, 1], Noemí Vila [a, b], Pedro Besada [a, b], Ernesto Cano [c], Carmen Terán [a, b, *]

[a] Departamento de Química Orgánica, Universidade de Vigo, 36310 Vigo, Spain
[b] Instituto de Investigación Biomédica (IBI), Universidade de Vigo, 36310 Vigo, Spain
[c] Departamento de Farmacoloxía, Universidade de Santiago de Compostela, 15782 Santiago de Compostela, Spain

ARTICLE INFO

Article history:
Received 7 November 2014
Received in revised form
27 February 2015
Accepted 28 February 2015
Available online 3 March 2015

Keywords:
Pyridazinone
3-Alkylfuran
Singlet oxygen
Butenolide
Bicyclic lactone
Platelet aggregation inhibitors

ABSTRACT

New series of pyridazinone derivatives (**4**, **5** and **6**) were synthesized in good yields following a synthetic strategy based on singlet oxygen oxidation of alkyl furans, in which a suitable $\beta(\alpha)$-substituted γ-hydroxybutenolide (**10** or **11**) or a bicyclic lactone (**12** or **13**) was the key intermediate. The synthesized compounds were tested in vitro as antiplatelet agents and some of them (compounds **4b**, **4d** and **5b**) exhibited potent inhibitory effects on collagen-induced platelet aggregation with IC_{50} values in the low μM range. Studies performed with the most active compound of these series (**4b**) demonstrated its lack of activity as inhibitor of platelet aggregation induced by other agonists as thrombin, ionomycin or U-46619 suggesting a selective action on the biochemical mechanisms triggered by collagen in the platelets.

1. Introduction

Cardiovascular diseases (CVDs), in particular coronary heart disease and stroke, are a leading cause of mortality in developed countries. According to the World Health Organization (WHO) 17 million people die every year by CVDs, accounting for almost one third of deaths worldwide per year [1]. In addition, CVDs are not only responsible for high morbidity and mortality, but negatively impact the quality of life of a huge amount of people across the world. Thrombus formation and hypertension are the most common risk factors for myocardial infarction and stroke. The pivotal role played by platelet activation in the physiopathology of thrombotic and ischemic diseases and the current limitations of antiplatelet therapy have prompted the search for new antiplatelet agents acting over novel therapeutic targets [2].

The 6-(aryl or heteroaryl)pyridazinone derivatives show a wide pharmacological profile that includes interesting properties on cardiovascular system, such as cardiotonic effects [3], antihypertensive activity [4] and platelet aggregation inhibition [5], being zardaverine, levosimendan and motapizone some characteristic drugs [5a,6] (Fig. 1).

The presence of the aryl (heteroaryl) group at C6 was considered essential when the cardiovascular effects are associated with phosphodiesterase III (PDE III) inhibition [5a,7]. Recently, several pyridazinone derivatives, in which the aryl or heteroaryl group at C6 was removed or replaced (compounds **1** and **2**, Fig. 2), were described as potential antihypertensive agents or platelet aggregation inhibitors whose activity is not related with inhibition of PDE [8,9].

In relation to cardiovascular drug research based on pyridazinone moiety we have recently reported a series of 2,6-disubstituted analogs with good antiplatelet properties. These pyridazinone derivatives have an alkyl chain of varying magnitude (1, 2, or 3 carbon atoms) functionalized with alcohol or ether groups in C6, and substituted or not with different lipophilic fragments in N2 (compounds **3**, Fig. 2). Most of the previously designed compounds inhibited platelet aggregation induced by collagen in the low μM range (1.80–69.6), being silyl ethers and N,O-dibenzyl derivatives the most active compounds. Many of them also showed a moderate vasorelaxant effect [10].

The interesting antiaggregatory properties showed by the 2,6-disubstituted pyridazinones **3** led us to continue with these studies in order to establish structure–activity relationships.

* Corresponding author. Departamento de Química Orgánica, Universidade de Vigo, 36310 Vigo, Spain.
E-mail address: mcteran@uvigo.es (C. Terán).
[1] T.C. and M.C.C.-L. contributed equally to this work.

http://dx.doi.org/10.1016/j.ejmech.2015.02.061
0223-5234/

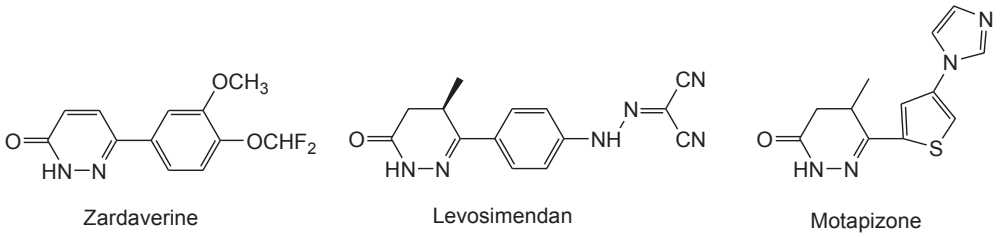


Fig. 1. Some cardiovascular drugs with the characteristic structure of 6-(aryl or heteroaryl)pyridazinone.

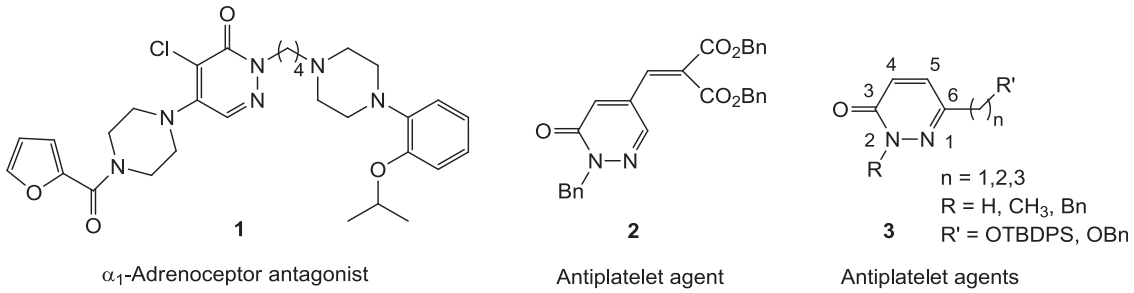


Fig. 2. Several pyridazinone derivatives with cardiovascular activity devoid of C6-(aryl or heteroaryl) group.

Therefore, we have synthesized and evaluated three new series of pyridazinone derivatives that have been designed considering two kinds of structural modifications, a positional change of the alkyl chain, which in the new analogs was placed at C5 or C4 of the pyridazinone ring (compounds **4** and **5** respectively, Fig. 3), and a reduction in conformational flexibility through the alkyl chain incorporation in different size rings resulting in bicyclic analogs **6** (Fig. 3).

2. Results and discussion

2.1. Chemistry

The pyridazinone derivatives studied in this work (compounds **4**, **5** and **6**) were synthesized according to the general procedures outlined in Schemes 1–3. Thus, the pyridazinone nucleus for monocyclic derivatives (**4** and **5**) was built by reaction of the $\beta(\alpha)$-substituted γ-hydroxybutenolide (**10** or **11**) with hydrazine or benzylhydrazine (Scheme 2), whereas for obtaining the fused pyridazinone core (**6**) a bicyclic lactone (**12** or **13**) was selected as the precursor suitable to react with hydrazine (Scheme 3).

The 4-hydroxybutenolides **10** and **11** were prepared in 3 steps from the commercially available ethyl 3-furoate (**7**) via oxidation with singlet oxygen, as shown in Scheme 1. Reduction of ester **7** with lithium aluminum hydride ($LiAlH_4$) in diethyl ether afforded alcohol **8** [11] (93% yield) which was protected as *tert*-butyldiphenylsilyl ether (compound **9** [12], yield 92%) in order to be submitted to singlet oxygen oxidation. Several protocols have been reported for the regioselective transformation of 3-substituted furans into α-substituted or β-substituted γ-hydroxybutenolides, via oxidation with singlet oxygen, in which a base-promoted regiocontrol was observed. In all cases, the oxidation was carried out in methanol or dichloromethane (DCM) at −78 °C in the presence of the suitable base. Thus, when the reaction is accomplished with some bulky bases, such as diisopropylethyl amine (DIPEA, Hünig's base), the β-substituted γ-hydroxybutenolides were the major products as a result of the sterically most favorable rearrangement [13,14]. The selective synthesis of the corresponding α-substituted γ-hydroxybutenolides is also possible by switching the base, for instance by using 1,8-diazabicyclo[5.4.0]undec-7-ene (DBU) or tetrabutylammonium fluoride (TBAF). However, in this last strategy the reported results are extremely substrate-dependent [14,15]. In our case, oxidation of the alkylfuran **9** with singlet oxygen in the presence of the Hüning's base and methanol gave a mixture of regioisomers **10** and **11** in a 4:1 ratio and quantitative yield (Method A). Moreover, the proportion of the α-substituted γ-hydroxybutenolide **11** was increased with the use of DBU in DCM obtaining the mixture of **10** and **11** in 1:1.25 ratio and excellent yield (Method B, 96%). Purification by column chromatography afforded enough quantities of both regioisomers in order to identify them. Characterization of butenolides **10** and **11** was performed analyzing the differences found in their ^{1}H NMR data and specifically in the multiplicity of the methylene hydrogen atoms. These protons, which for the α-substituted butenolide **11** resonate as a multiplet centered at 4.48 ppm, appear fully differentiated for the β-substituted compound **10** as two doublet of doublets at 4.46 and 4.60 ppm, (J = 17.8 and 2.0 Hz) respectively. It is noteworthy that in the β-substituted butenolide (**10**) the oxygen atom of the side chain is close to the C5 hydroxyl group allowing the formation of a stable 6-membered ring through hydrogen bonding interactions which would explain the multiplicity differences observed.

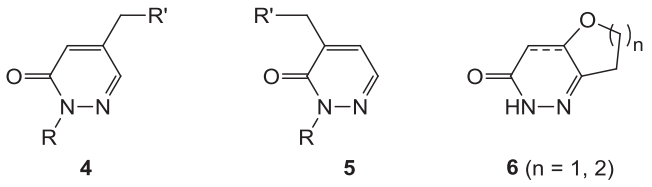
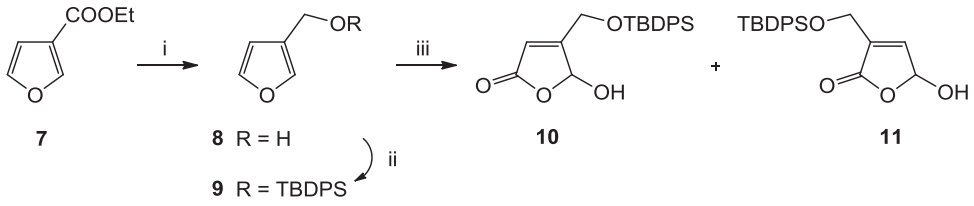
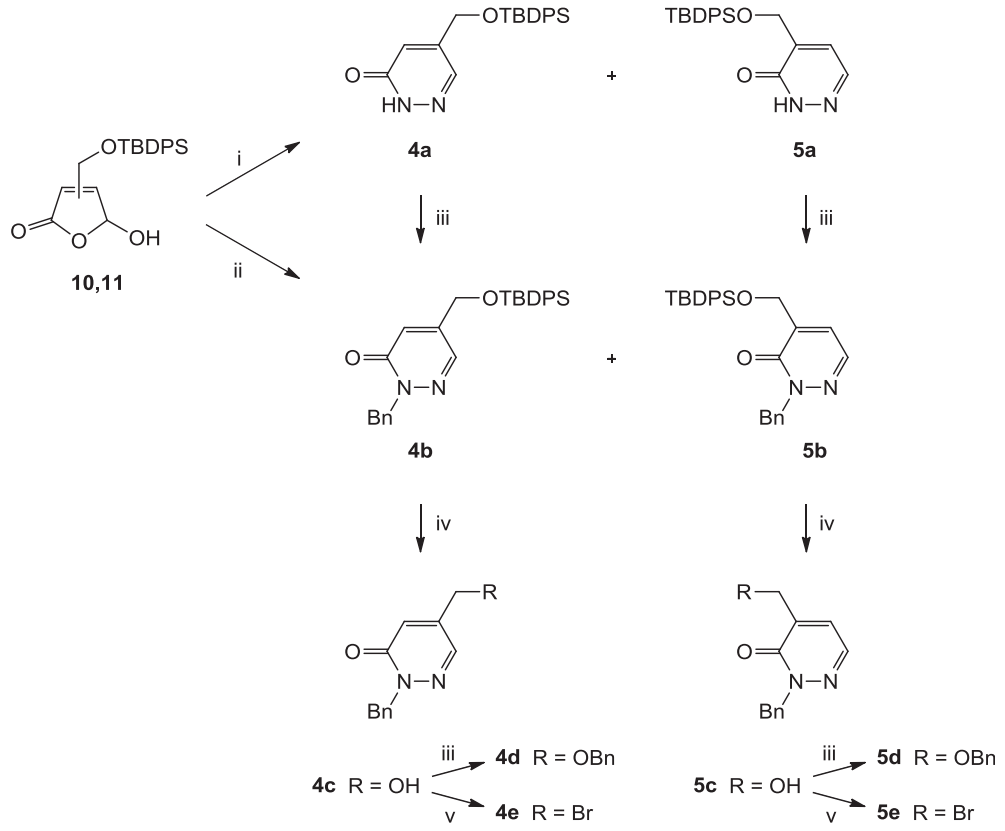


Fig. 3. General structure of pyridazinone analogs studied.

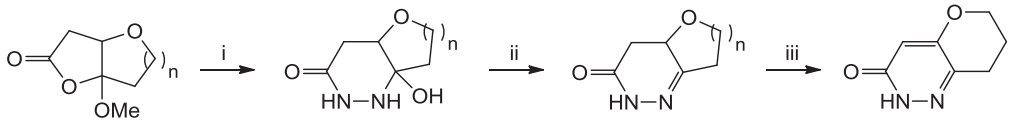
Treatment of a mixture containing **10** and **11** with hydrazine monohydrate in ethanol at reflux gave the pyridazinones **4a** and **5a** which were easily isolated and purified by column chromatography (Scheme 2). The structure of pyridazinone isomers **4a**–**5a** was established by the single crystal X-ray studies (Figs. 4 and 5) [16,17].



Scheme 1. Reagents and conditions: (i) $LiAlH_4$, Et_2O, r.t., 45 min, 93%; (ii) TBDPSCl, imidazole, DMF, r.t., 20 h, 92%; (iii) O_2, hν, rose bengal, DIPEA, MeOH, −78 °C, 5 h, (**10** and **11** ratio 4:1, Method A) or DBU, CH_2Cl_2, −78 °C, 2 h (**10** and **11** ratio 1:1.5, Method B).



Scheme 2. Reagents and conditions: (i) NH_2NH_2, EtOH, reflux, 4 h, 56% (**4a** from **9**), 5% (**5a** from **9**); (ii) $BnNHNH_2 \cdot 2HCl$, Et_3N, EtOH, reflux, 7 h (Method C), 46% (**4b** from **9**), 7% (**5b** from **9**); (iii) NaH, BnBr, Bu_4NI, THF, r.t., 24 h (Method D), 68% (**4b**), 62% (**5b**), 81% (**4d**), 56% (**5d**); (iv) TBAF, THF, r.t., 30 min, 87% (**4c**), 75% (**5c**); (v) CBr_4, Ph_3P, CH_2Cl_2, reflux, 1.5 h, 58% (**4e**), 84% (**5e**).



Scheme 3. Reagents and conditions: (i) NH_2NH_2, EtOH, reflux, 5 h, 97% (**14**), 98% (**15**); (ii) $BF_3 \cdot OEt_2$, CH_2Cl_2, rt, 14 h or 1.5 h, 96% (**6a**), 73% (**6b**); (iii) MnO_2, DMF, reflux, 5 h, 47%.

The N-benzylpyridazinones **4b**–**5b** were obtained in a similar way by reaction of the mixture containing **10** and **11** with benzylhydrazine dihydrochloride (Method C) and their structures were determined from NMR spectroscopy by comparison with **4a**–**5a** data, in particular taking into account the *J* values of the ring proton signals (see experimental part). Regiochemistry of compounds **4b**–**5b** was further confirmed by benzylation of **4a**–**5a** with benzyl bromide at room temperature (Method D) [18]. Moreover, compounds **4b**–**5b** by treatment with TBAF provided the hydroxymethyl derivatives **4c**–**5c**, which were transformed into the N,O-dibenzyl analogs **4d**–**5d** using the standard procedure above mentioned. The bromomethyl derivatives **4e**–**5e** were obtained in moderate to good yields (58–84%) by refluxing of hydroxymethyl derivatives **IVc**–**Vc** with carbon tetrabromide and triphenylphosphine in DCM [19] (Scheme 2).

The bicyclic lactones **12** and **13**, key intermediates to obtain the

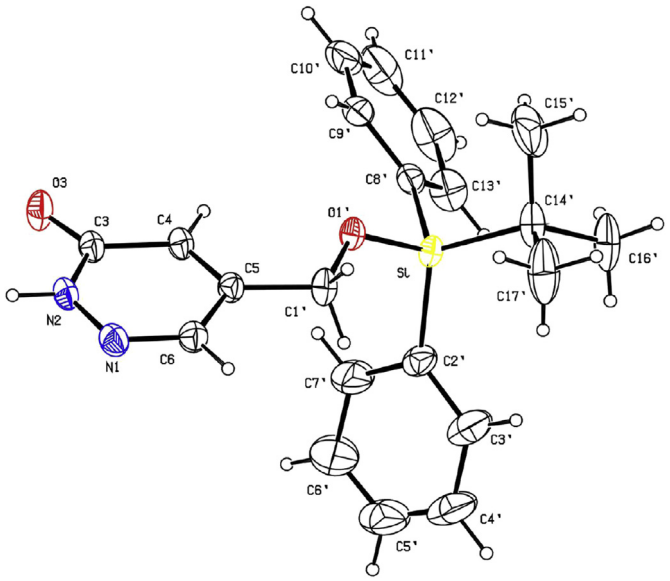


Fig. 4. X-ray crystal structure of compound **4a**. Displacement ellipsoids are shown at the 20% probability level.

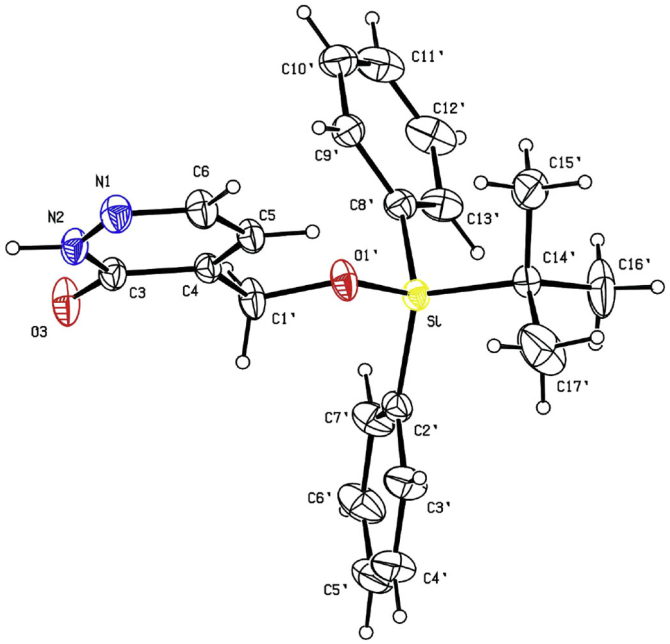


Fig. 5. X-ray crystal structure of compound **5a**. Displacement ellipsoids are shown at the 20% probability level.

fused pyridazinone core analogs **6**, were synthesized from the corresponding γ-substituted γ-methoxy butenolide by treatment with TBAF in THF, trough an intramolecular Michael addition, as previously reported [20,21]. Treatment of **12** or **13** with hydrazine monohydrate in ethanol at reflux afforded the bicyclic hydroxyhydrazides **14** or **15** respectively in excellent yields. Then they were easily dehydrated by reaction with boron trifluoride diethyl etherate ($BF_3{\cdot}EtO_2$) in DCM at 0 °C to give the desired furopyridazinone **6a** (yield 96%) and pyranopyridazinone **6b** (yield 73%) (Scheme 3). Moreover, with the idea of getting a direct transformation of the bicyclic lactones into the fused pyridazinone derivatives **6**, a solution of compound **13** and hydrazine hydrate in ethanol was refluxed with a catalytic amount of *p*-toluenesulfonic acid. However, these conditions led to the pyran ring-opening via the pyridazinone nucleus aromatization, resulting in the monocyclic derivative 6-(3-hydroxypropyl)pyridazin-3(2H)-one instead of **6b**. Finally, oxidation of **6b** with manganese dioxide in DMF at reflux gave rise to the bicyclic unsaturated pyranopyridazinone analog **6c** in moderate yield (47%).

2.2. Antiplatelet activity

Platelet aggregation studies of target compounds were performed in washed human platelets following the Born's turbidimetric method [22] and using collagen (2–4 μg/mL) as platelet aggregation inducer [23]. A total of thirteen new pyridazinone derivatives (**4a**–**e**, **5a**–**e** and **6a**–**c**) were tested in order to determine if the new monocyclic and bicyclic analogs show similar antiplatelet activity to that exhibited by the C6-substituted pyridazinone derivatives (**3a**–**c**) previously reported [10].

Table 1 shows the IC_{50} values against collagen for the new analogs and **3a**–**c** derivatives [10]. They were obtained from experiments conducted at concentration intervals of 0.5–5 μM or 10–100 μM, depending on the compound.

The obtained results revealed that five of the new studied derivatives displayed high potency against collagen-induced platelet aggregation.

As can be seen from Table 1 data, the N-benzyl silyl ethers **4b**–**5b** and the N,O-dibenzyl derivatives **4d**–**5d** exhibited the best activity, with IC_{50} values of 0.55 ± 0.08 (**4b**), 1.31 ± 0.37 (**5b**), 3.75 ± 0.48 (**4d**) and 17.52 ± 2.53 (**5d**), which is consistent with the results previously reported for the C6-substituted analogs (compounds **3**).

In addition, in the fused-pyridazinone series, the furopyridazinone derivative **6a** showed low activity (IC_{50} value of 70.57 ± 15.55 μM) as compared with the corresponding monocyclic analogs [10].

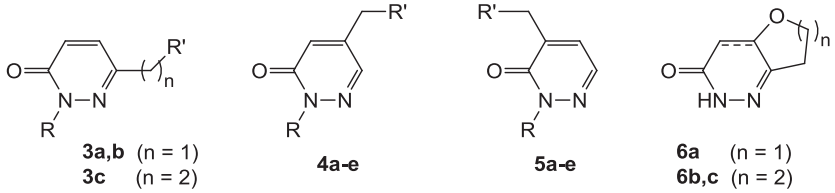
It should be noted that in all monocyclic analogs, both the C6-substituted previously described [10] and the C5 or C4 substituted derivatives now synthesized, the presence of some lipophilic fragments, such as a tertbutyldiphenylsilyl or a benzyl group in the alkyl chain and a benzyl group in N2, was favorable for the activity.

Therefore, the obtained results showed that antiplatelet activity was unaffected by a positional change of the lateral chain in the pyridazinone core. However, the activity was significantly diminished when the alkyl chain was incorporated in a ring.

In order to find any significant information about the molecular effect of tested pyridazinone derivatives on platelet biology, subsequent pharmacological studies were conducted with **4b**, the most active compound of this series. Firstly, it has been found that a supramaximal dose of **4b** (20 μM) was unable to significantly inhibit platelet aggregation induced by different platelet aggregatory stimulants, such as thrombin, U-46619 (a stable thromboxane A2 analog) or the calcium ionophore ionomycin (Fig. 6). These results suggested a selective action of **4b** on the biochemical mechanisms triggered by collagen in the platelets.

Collagen interacts with platelets through direct and indirect mechanisms, and although several targets have been involved, two main surface receptors have been identified, the integrin α2β1, with a major role in adhesion and platelet anchoring and the Immunoglobulin (Ig) superfamily member glycoprotein VI (GPVI), principally responsible for signaling and platelet activation [24]. GPVI-selective agonists, such as convulxin and collagen-related peptide (CRP), are strong inducers of platelet aggregation. It has been found that **4b** completely inhibits platelet aggregation caused by the agonist CRP-XL (cross-linked form of CRP; Fig. 6), indicating that

Table 1
Antiplatelet activity data against collagen induced platelet aggregation.



Compound	R	R′	IC_{50} μM[a]
3a	Bn	OTBDPS	4.6 ± 0.4[b]
3b	Bn	OBn	8.0 ± 0.4[b]
3c	H	OTBDPS	3.87 ± 0.38[b]
4a	H	OTBDPS	*
4b	Bn	OTBDPS	0.55 ± 0.08
4c	Bn	OH	>100
4d	Bn	OBn	3.75 ± 0.48
4e	Bn	Br	>100
5a	H	OTBDPS	*
5b	Bn	OTBDPS	1.31 ± 0.37
5c	Bn	OH	>100
5d	Bn	OBn	17.52 ± 2.53
5e	Bn	Br	>100
6a	–	–	70.57 ± 15.55
6b	–	–	>100
6c	–	–	>100

*Induces platelet aggregation at concentration lower than 100 μM.
[a] Values were expressed as means ± S.E.M (n = 4).
[b] Values obtained from ref. [10].

this pyridazinone derivative acts on some of the steps involved in platelet activation via GPVI pathway. In addition, as the CRP is able to induce full aggregation in the presence of inhibitors of cyclo-oxygenase [25,26], our results demonstrate that **4b** is not a COX inhibitor, one of the mechanisms previously suggested for explaining the activity of this kind of pyridazinone analogs [10].

Stimulation of platelet by any agonist requires activation of the platelet integrin adhesion receptor αIIbβ3 (GP IIb/IIIa) to bind fibrinogen and link adjacent platelets together in an aggregate [27]. Binding of platelet agonists to their plasma membrane receptors promote cellular responses leading to the change of αIIbβ3 from the resting state to the activated state via a conformational change in the extracellular domain, which enhances its affinity for soluble ligands like fibrinogen; these molecular events are referred to as "inside-out" signaling [28]. Ligand binding to αIIbβ3 becomes progressively irreversible by integrin clustering, triggering a number of cellular events that include cytoskeletal reorganization, changes in enzymatic activities and stabilization of large platelet aggregates; these molecular events are referred as "outside-in" signaling [29].

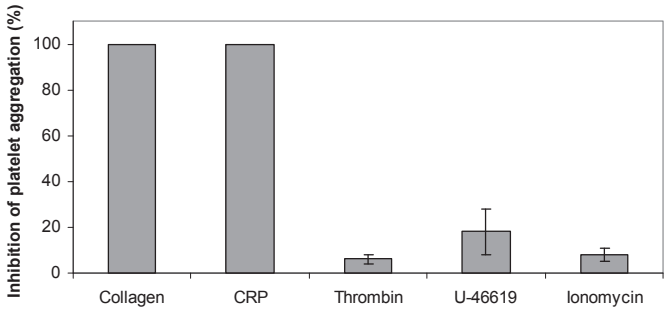


Fig. 6. Effects of supramaximal dose of **4b** on platelet aggregation induced by different platelet activators. Data represent mean ± standard error of the mean (s.e.m) of at least four experiments in duplicate; for Collagen and CRP s.e.m was 0.

Using flow cytometry and PAC-1 [30] (a monoclonal antibody which recognizes the active conformation of GP IIb/IIIa receptor) the level of Gp IIb/IIIa activation following platelet stimulation, and hence the effect of a particular compound on the cellular events that cause conformational changes of GpIIb/IIIa (i.e. the "inside-out" signaling), can be measured. Thus, we have found that dose of **4b** causing complete inhibition of platelet aggregation only partially inhibits the GP IIb/IIIa activation (Fig. 7). Since that (i) a conformational change in αIIbβ3 is crucial for platelet activation [30], (ii) inhibition of platelet aggregation correlates well with percentage blockage of GPIIb/IIIa [31] and (iii) total inhibition of this integrin causes the failure of platelets to aggregate [32], these results showing a partial inhibition suggest that **4b** should act on both "inside-out" and "outside-in" signaling.

Stimulation of GPVI by collagen or CRP induces platelet activation through a tyrosine kinase–based signaling pathway. GPVI is non-covalently associated with the Fc Receptor γ chain (FcRγ) [33], which acts as the signal-transducing subunit of the receptor [34]. Ligand binding causes cross-linking of GPVI leading to tyrosine phosphorylation of the immunoreceptor tyrosine-based activation motif (ITAM) within the FcRγ cytoplasmic domain by Src-family tyrosine kinases (mainly Lyn and Fyn) bound to the cytoplasmic domain of GPVI [35]. The ITAM phosphorylation gives rise to binding and activation of the tyrosine kinase Syk, which phosphorylates downstream targets and generates a large signaling complex involving adapter proteins, Tec family tyrosine kinases, GTP-exchange factors, small GTPase Rac1, phosphatidylinositol 3-kinase isoforms and phospholipase Cγ2 as main effector protein [36].

Therefore, the effect of 4b on the increase of phosphotyrosine induced by CRP-XL on platelet proteins through an immunodot blot assay was investigated, using a platelet lysate and the anti-phosphotyrosine specific antibody 4G10. The obtained results showed that compound **4b** induces a decrease in total amount of

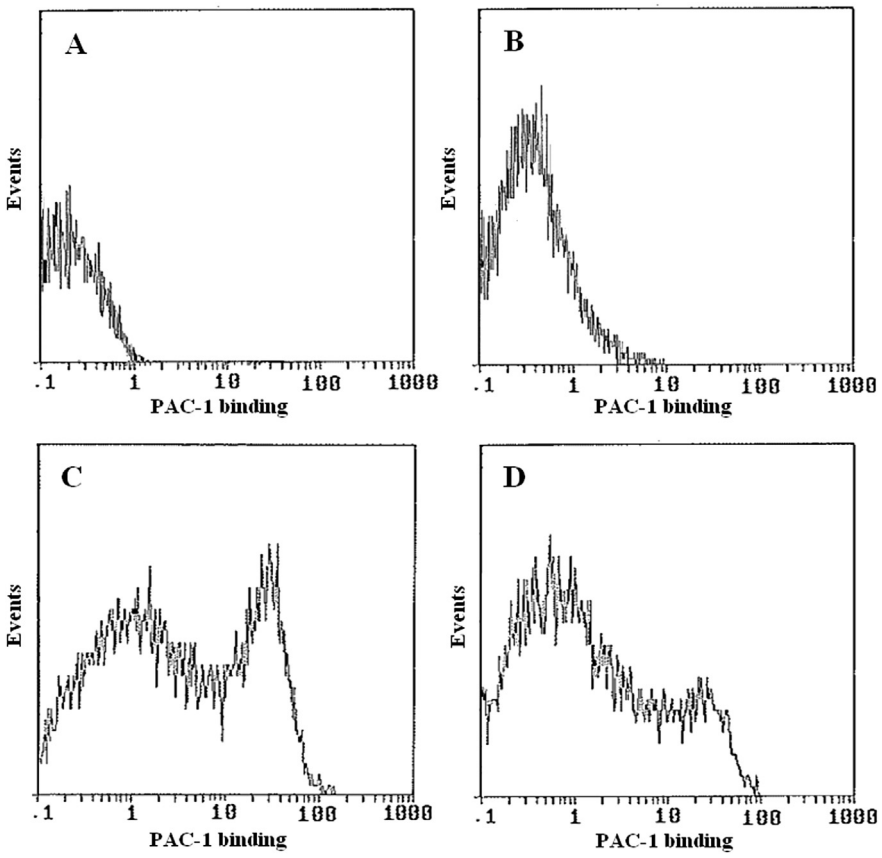


Fig. 7. Effect of compound **4b** on GpIIb/IIIa activation induced by CRP-XL. A: RGDS treated platelets (non-specific binding of PAC-1). B: non-stimulated platelets (basal conditions). C: CRP-XL stimulated platelets. D: platelets treated with compound **4b** and stimulated with CRP-XL.

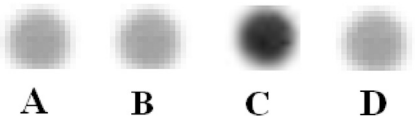


Fig. 8. Effect of compound **4b** on protein tyrosine phosphorylation in platelets stimulated by CRP-XL. A: non-stimulated platelets (basal conditions). B: platelets treated with compound **4b** (10 μM). C: platelets stimulated by CRP-XL (5 μg/mL). D: platelets treated with compound **4b** (10 μM) and stimulated by CRP-XL (5 μg/mL). Representative result from four experiments in duplicate.

phosphotyrosine residues of platelet proteins in a dose-dependent manner, which is well correlated with antiplatelet effect (Fig. 8).

Thus, the decrease on tyrosine phosphorylation displayed by **4b** could explain the effectiveness of this compound against the activation of platelets by collagen and CRP. Bearing in mind that the phosphorylation state of any protein is the result of the balance between phosphorylation/dephosphorylation processes, the decrease in the level of tyrosine phosphorylation caused by **4b** can be explained both by a decrease in the tyrosine–kinase activity and a stimulation in tyrosine–phosphatase activity. Our data provide a starting point for a new research work in order to know the proteins affected by the action of tested compounds as well as the different platelet tyrosine kinases or tyrosine phosphatases that could be involved.

3. Conclusions

Three new series of pyridazinone derivatives (**4**, **5** and **6**) were synthesized in good yields following a synthetic strategy based on singlet oxygen oxidation of alkyl furans, in which a suitable $\beta(\alpha)$-substituted γ-hydroxybutenolide or a bicyclic lactone was the key intermediate. Three of these new pyridazinone analogs displayed high activity against collagen-induced platelet aggregation. Studies performed with the most active compound of these series (**4b**) demonstrated its lack of activity as inhibitor of platelet aggregation induced by other agonists as thrombin, ionomycin or U-46619. However, when CRP was used as platelet aggregation inducer, compound **4b** partially decreased the conformational change of GPIIb/IIIa receptor. In addition, compound **4b** also inhibited the increase in the phosphotyrosine content of platelet proteins caused by activation of GPVI receptor.

Accordingly, the inhibition of collagen-induced platelet aggregation caused by the target pyridazinone analogs can be due to its ability to decrease tyrosine phosphorylation of some platelet proteins.

4. Experimental section

4.1. Chemistry

All starting materials and common laboratory chemicals were purchased from commercial sources and used without further purification. All solvents were distilled and dried according to standard procedures. ^{1}H and ^{13}C NMR spectra were recorded on a Bruker ARX400 instrument, using TMS as internal standard [chemical shifts (δ) in ppm, J in Hz]. The assignment of the signals was performed by COSY, DEPT, HSQC experiments. High resolution mass spectra were recorded using a Bruker microTOF focus spectrometer. X-ray analysis was performed on a Bruker SMART 1000

CCD diffractometer. Silica gel (Merck 60, 230–400 mesh) was used for flash chromatography (FC). Analytical TLC was performed on plates precoated with silica gel (Merck 60 F254, 0.25 mm).

4.1.1. 3-Furylmethanol (**8**)

To a solution of ethyl 3-furoate (**7**) (4.00 g, 28.5 mmol) in Et_2O (140 mL) was added $LiAlH_4$ (1.62 g, 42.8 mmol) in small portions at 0 °C. The reaction mixture was stirred at room temperature for 45 min, followed by quenching with H_2O (2 mL), NaOH 1 M (2 mL) and H_2O (6 mL) at 0 °C. The resulting white precipitate was filtered off and the solvent was removed under reduced pressure affording compound **8** (2.60 g, 93%) as a yellowish oil. $R_f = 0.2$ (hexane/ethyl acetate, 4:1). ^{1}H NMR ($CDCl_3$): $\delta = 7.42–7.41$ (m, 1H, H2), 7.40–7.39 (m, 1H, H5), 6.44–6.42 (m, 1H, H4), 4.55 (s, 2H, CH_2), 1.81 (br s, 1H, OH). ^{13}C NMR ($CDCl_3$): $\delta = 143.3$ (C5), 139.9 (C2), 125.1 (C3), 109.9 (C4), 56.1 (CH_2). HRMS-EI: *m/z* $[M]^+$ calcd for $C_5H_6O_2$: 98.0368, found: 98.0371.

4.1.2. 3-(tert-Butyldiphenylsilyloxymethyl)furan (**9**)

To a solution of compound **8** (2.60 g, 26.5 mmol) in DMF (30 mL) was added imidazole (2.49 g, 36.5 mmol) and TBDPSCl (9.5 mL, 36.5 mmol). The reaction mixture was stirred at room temperature for 20 h, quenched with H_2O (50 mL) and extracted with Et_2O (3×50 mL). The combined organic phases were dried over Na_2SO_4, filtered and the solvent was evaporated to dryness. The residue was purified by column chromatography on silica gel (hexane/ethyl acetate, 99:1) to obtain compound **9** (8.20 g, 92%) as a yellowish oil. $R_f = 0.6$ (hexane/ethyl acetate, 4:1). ^{1}H NMR ($CDCl_3$): $\delta = 7.72–7.69$ (m, 4H, H-Ph), 7.46–7.37 (m, 7H, H-Ph, H5), 7.33–7.32 (m, 1H, H2), 6.35–6.33 (m, 1H, H4), 4.63 (s, 2H, CH_2), 1.08 (s, 9H, $(CH_3)_3$). ^{13}C NMR ($CDCl_3$): $\delta = 143.1$ (C5), 139.5 (C2), 135.7 (CH-Ph), 133.7 (C-Ph), 129.9 (CH-Ph), 127.9 (CH-Ph), 125.5 (C3), 109.7 (C4), 58.4 (CH_2), 26.9 ($(CH_3)_3$), 19.4 ($C(CH_3)_3$). HRMS-ESI: *m/z* $[M+H]^+$ calcd for $C_{21}H_{25}O_2Si$: 337.16183, found: 337.16241.

4.1.3. 4-(tert-Butyldiphenylsilyloxymethyl)-5-hydroxy-5H-furan-2-one (**10**) and 3-(tert-butyldiphenylsilyloxymethyl)-5-hydroxy-5H-furan-2-one (**11**)

Method A: To a solution of compound **9** (3.00 g, 8.92 mmol) in MeOH (40 mL) was added *N,N*-diisopropylethylamine (7.1 mL, 40.2 mmol), rose bengal (15 mg). Then it was purged with O_2 for 1 h. The reaction mixture was irradiated with a 200 W lamp and stirred under oxygen atmosphere for 5 h at −78 °C. The solvent was evaporated, the residue was dissolved in CH_2Cl_2 (40 mL) and 0.12 M oxalic acid in H_2O (350 mL) was added. The mixture was stirred for 30 min at room temperature and extracted with CH_2Cl_2 (3×100 mL). The combined organic phases were dried over Na_2SO_4, filtered and the solvent was evaporated to dryness. The residue was rapidly passed through a column chromatography on silica gel (hexane/ethyl acetate, 2:1) to afford compound **10** (56 mg) as a colorless oil, then a mixture containing **10** and **11** (3.61 g) in a 4:1 ratio and finally compound **11** (20 mg) as a colorless oil. **Method B**: To a solution of compound **9** (300 mg, 0.89 mmol) in CH_2Cl_2 (20 mL) was added 1,8-diazabicyclo[5.4.0]undec-7-ene (0.27 mL, 1.78 mmol), rose bengal (5 mg). Then it was purged with O_2 for 1 h. The reaction mixture was irradiated with a 200 W lamp and stirred under oxygen atmosphere for 2 h at −78 °C. The mixture was allowed to warm at 0 °C, washed with HCl 1 M (15 mL) and extracted with CH_2Cl_2 (2×15 mL). The organic phase was dried over Na_2SO_4 and evaporated to dryness and the residue was purified by column chromatography on silica gel (hexane/ethyl acetate, 4:1) to afford a mixture containing **10** and **11** (299 mg) in a 1:1.25 ratio and then compound **11** (15 mg) as a colorless oil. Compound **10**: $R_f = 0.3$ (hexane/ethyl acetate, 2:1). ^{1}H NMR ($CDCl_3$): $\delta = 7.67–7.63$ (m, 4H, H-Ph), 7.47–7.38 (m, 6H, H-Ph), 6.18–6.16 (m, 1H, H3), 5.99 (s, 1H, H5), 4.69 (br s, 1H, OH), 4.60 (dd, 1H, $J = 17.8$, 2.0 Hz, CH_2), 4.46 (dd, 1H, $J = 17.8$, 2.0 Hz, CH_2), 1.09 (s, 9H, $(CH_3)_3$). ^{13}C NMR ($CDCl_3$): $\delta = 171.2$ (C2), 168.7 (C4), 135.6 (CH-Ph), 132.5 (C-Ph), 130.3 (CH-Ph), 128.1 (CH-Ph), 117.7 (C3), 97.4 (C5), 60.0 (CH_2), 26.8 ($(CH_3)_3$), 19.3 ($C(CH_3)_3$). HRMS-ESI: *m/z* $[M+H]^+$ calcd for $C_{21}H_{25}O_4Si$: 369.15166, found: 369.15162. Compound **11**: $R_f = 0.2$ (hexane/ethyl acetate, 2:1). ^{1}H NMR ($CDCl_3$): $\delta = 7.66–7.62$ (m, 4H, H-Ph), 7.47–7.36 (m, 6H, H-Ph), 7.15–7.13 (m, 1H, H4), 6.11 (s, 1H, H5), 4.49–4.47 (m, 2H, CH_2), 1.09 (s, 9H, $(CH_3)_3$). ^{13}C NMR ($CDCl_3$): $\delta = 169.5$ (C2), 143.7 (C4), 138.6 (C3), 135.6 (CH-Ph), 132.7 (C-Ph), 130.2 (CH-Ph), 128.1 (CH-Ph), 97.3 (C5), 58.8 (CH_2), 26.9 ($(CH_3)_3$), 19.3 ($C(CH_3)_3$). HRMS-ESI: *m/z* $[M+H]^+$ calcd for $C_{21}H_{25}O_4Si$: 369.15166, found: 369.15261.

4.1.4. 5-(tert-Butyldiphenylsilyloxymethyl)pyridazin-3(2H)-one (**4a**) and 4-(tert-butyldiphenylsilyloxymethyl)pyridazin-3(2H)-one (**5a**)

To a solution of a mixture containing **10** and **11** (1.09 g) in a 4:1 ratio in ethanol (15 mL) was added hydrazine monohydrate (0.3 mL, 6.18 mmol). After the reaction mixture was stirred under reflux for 4 h, the solvent was removed and the residue was purified by column chromatography on silica gel (hexane/ethyl acetate, 4:1) to afford **5a** (45 mg, 5% from **9**) as a colorless oil and then **4a** (508 mg, 56% from **9**) as a white solid. Compound **4a**: $R_f = 0.2$ (hexane/ethyl acetate, 2:1). ^{1}H NMR ($CDCl_3$): $\delta = 12.91$ (br s, 1H, NH), 7.72 (d, 1H, $J = 1.9$ Hz, H6), 7.69–7.65 (m, 4H, H-Ph), 7.49–7.38 (m, 6H, H-Ph), 7.09–7.07 (m, 1H, H4), 4.61 (d, 2H, $J = 1.3$ Hz, CH_2), 1.12 (s, 9H, $(CH_3)_3$). ^{13}C NMR ($CDCl_3$): $\delta = 162.8$ (C3), 147.3 (C5), 136.4 (C6), 135.6 (CH-Ph), 132.4 (C-Ph), 130.3 (CH-Ph), 128.1 (CH-Ph), 124.5 (C4), 62.1 (CH_2), 26.8 ($(CH_3)_3$), 19.4 ($C(CH_3)_3$). HRMS-ESI: *m/z* $[M+H]^+$ calcd for $C_{21}H_{25}N_2O_2Si$: 365.16798, found: 365.16806. Compound **5a**: $R_f = 0.3$ (hexane/ethyl acetate, 2:1). ^{1}H NMR ($CDCl_3$): $\delta = 12.32$ (br s, 1H, NH), 7.90 (d, 1H, $J = 4.0$ Hz, H6), 7.67–7.63 (m, 4H, H-Ph), 7.61–7.58 (m, 1H, H5), 7.46–7.35 (m, 6H, H-Ph), 4.77 (d, 2H, $J = 1.7$ Hz, CH_2), 1.14 (s, 9H, $(CH_3)_3$). ^{13}C NMR ($CDCl_3$): $\delta = 161.1$ (C3), 143.7 (C4), 137.9 (C6), 135.5 (CH-Ph), 132.7 (C-Ph), 130.2 (CH-Ph), 128.1 (CH-Ph), 126.5 (C5), 60.5 (CH_2), 27.0 ($(CH_3)_3$), 19.5 ($C(CH_3)_3$). HRMS-ESI: *m/z* $[M+H]^+$ calcd for $C_{21}H_{25}N_2O_2Si$: 365.16798, found: 365.16804.

4.1.5. 2-Benzyl-5-(tert-butyldiphenylsilyloxymethyl)pyridazin-3(2H)-one (**4b**) and 2-benzyl-4-(tert-butyldiphenylsilyloxymethyl) pyridazin-3(2H)-one (**5b**)

Method C: To a solution of a mixture containing **10** and **11** (531 mg) in a 4:1 ratio in ethanol (15 mL) was added benzylhydrazine dihydrochloride (562 mg, 2.88 mmol) and Et_3N (0.6 mL, 4.30 mmol) and the reaction mixture was stirred under reflux for 7 h. After the solvent was removed, CH_2Cl_2 (10 mL) and H_2O (10 mL) were added and the mixture was extracted with CH_2Cl_2 (3×10 mL). The combined organic phases were dried over Na_2SO_4, filtered and the solvent was evaporated to dryness. The residue was purified by column chromatography on silica gel (hexane/ethyl acetate, 9:1) to afford **5b** (43 mg, 7% from **9**) as a colorless oil and then **4b** (277 mg, 46% from **9**) as a yellowish oil. **Method D**: A solution of compound **4a** or **5a** (50 mg, 0.14 mmol) in THF (4 mL) was added, dropwise at 0 °C, to a suspension of NaH (9 mg, 0.21 mmol, 60% dispersion in mineral oil) in THF (4 mL). After the mixture was stirred at r.t. for 1 h, BnBr (35 μL, 0.29 mmol) and Bu_4NI (5 mg, 0.01 mmol) were added. The reaction mixture was stirred at r.t. for 24 h, followed by quenching with MeOH. The solvent was evaporated to dryness, and the residue was purified by column chromatography on silica gel (hexane/ethyl acetate, 9:1) to afford **4b** (44 mg, 68%) as a yellowish oil or **5b** (39 mg, 62%) as a yellowish oil. Compound **4b**: $R_f = 0.5$ (hexane/ethyl acetate, 2:1). ^{1}H NMR ($CDCl_3$): $\delta = 7.69–7.64$ (m, 5H, H6, H-Ph), 7.49–7.27 (m, 11H, H-Ph),

7.01–6.99 (m, 1H, H4), 5.34 (s, 2H, CH_2N), 4.57 (d, 2H, J = 1.5 Hz, CH_2O), 1.12 (s, 9H, $(CH_3)_3$). ^{13}C NMR ($CDCl_3$): δ = 160.6 (C3), 145.6 (C5), 136.4 (C-Ph), 135.5 (CH-Ph), 135.3 (C6), 132.3 (C-Ph), 130.2 (CH-Ph), 128.8 (CH-Ph), 128.7 (CH-Ph), 128.1 (CH-Ph), 128.0 (CH-Ph), 124.7 (C4), 61.8 (CH_2O), 58.9 (CH_2N), 26.8 ($(CH_3)_3$), 19.3 ($C(CH_3)_3$). HRMS-ESI: m/z $[M+H]^+$ calcd for $C_{28}H_{31}N_2O_2Si$: 455.21493, found: 455.21473. Compound **5b**: R_f = 0.6 (hexane/ethyl acetate, 2:1). ^1H NMR ($CDCl_3$): δ = 7.85 (d, 1H, J = 4.0 Hz, H6), 7.65–7.61 (m, 4H, H-Ph), 7.52–7.49 (m, 1H, H5), 7.45–7.26 (m, 11H, H-Ph), 5.29 (s, 2H, CH_2N), 4.73 (d, 2H, J = 1.5 Hz, CH_2O), 1.12 (s, 9H, $(CH_3)_3$). ^{13}C NMR ($CDCl_3$): δ = 159.5 (C3), 143.4 (C4), 136.7 (C6), 136.4 (C-Ph), 135.6 (CH-Ph), 132.9 (C-Ph), 130.1 (CH-Ph), 128.9 (CH-Ph), 128.7 (CH-Ph), 128.0 (2×(CH-Ph)), 125.3 (C5), 61.0 (CH_2O), 55.1 (CH_2N), 27.1 ($(CH_3)_3$), 19.5 ($C(CH_3)_3$). HRMS-ESI: m/z $[M+H]^+$ calcd for $C_{28}H_{31}N_2O_2Si$: 455.21493, found: 455.21371.

4.1.6. 2-Benzyl-5-hydroxymethylpyridazin-3(2H)-one (**4c**)

A solution of compound **4b** (247 mg, 0.54 mmol) in THF (5 mL) and TBAF 1 M in THF (0.8 mL, 0.81 mmol) was stirred at r.t. for 30 min. The solvent was evaporated to dryness, and the residue was purified by column chromatography on silica gel (ethyl acetate/methanol, 98:2) to afford **4c** (102 mg, 87%) as a yellowish oil. R_f = 0.5 (ethyl acetate/methanol, 95:5). ^1H NMR ($CDCl_3$): δ = 7.66–7.64 (m, 1H, H6), 7.36–7.31 (m, 2H, H-Ph), 7.30–7.24 (m, 3H, H-Ph), 6.87–6.84 (s, 1H, H4), 5.26 (s, 2H, CH_2N), 4.45 (s, 2H, CH_2O). ^{13}C NMR ($CDCl_3$): δ = 160.9 (C3), 146.9 (C5), 136.3 (C6), 136.1 (C-Ph), 128.7 (CH-Ph), 128.6 (CH-Ph), 128.1 (CH-Ph), 124.8 (C4), 60.7 (CH_2O), 55.2 (CH_2N). HRMS-ESI: m/z $[M+H]^+$ calcd for $C_{12}H_{13}N_2O_2$: 217.09715, found: 217.09782.

4.1.7. 2-Benzyl-4-hydroxymethylpyridazin-3(2H)-one (**5c**)

Compound **5c** (25 mg, 75%), yellowish oil, was obtained from **5b** (70 mg, 0.15 mmol) following the same procedure as for preparation of compound **4c** from **4b**. R_f = 0.6 (ethyl acetate/methanol, 95:5). ^1H NMR ($CDCl_3$): δ = 7.80 (d, 1H, J = 4.0 Hz, H6), 7.42–7.38 (m, 2H, H-Ph), 7.34–7.27 (m, 3H, H-Ph), 7.21 (dt, 1H, J = 4.0, 1.2 Hz, H5), 5.33 (s, 2H, CH_2N), 4.62 (d, 2H, J = 1.2 Hz, CH_2O). ^{13}C NMR ($CDCl_3$): δ = 160.5 (C3), 142.1 (C4), 136.8 (C6), 136.1 (C-Ph), 128.8 (CH-Ph), 128.7 (CH-Ph), 128.1 (CH-Ph), 126.4 (C5), 60.9 (CH_2O), 55.3 (CH_2N). HRMS-ESI: m/z $[M+H]^+$ calcd for $C_{12}H_{13}N_2O_2$: 217.09715, found: 217.09782.

4.1.8. 2-Benzyl-5-benzyloxymethylpyridazin-3(2H)-one (**4d**)

Compound **4d** (32 mg, 81%), yellowish oil, was obtained from **4c** (28 mg, 0.13 mmol) following the same procedure as for preparation of compound **4b** from **4a** (Method D). R_f = 0.3 (hexane/ethyl acetate, 2:1). ^1H NMR ($CDCl_3$): δ = 7.74 (d, 1H, J = 2.1 Hz, H6), 7.43–7.39 (m, 2H, H-Ph), 7.38–7.26 (m, 8H, H-Ph), 6.90–6.88 (m, 1H, H4), 5.31 (s, 2H, CH_2N), 4.59 (s, 2H, OCH_2Ph), 4.38 (d, 2H, J = 1.2 Hz, CH_2OBn). ^{13}C NMR ($CDCl_3$): δ = 160.4 (C3), 143.2 (C5), 137.1 (C-Ph), 136.3 (C-Ph), 136.0 (C6), 128.8 (CH-Ph), 128.7 (CH-Ph), 128.7 (CH-Ph), 128.3 (CH-Ph), 128.1 (CH-Ph), 127.9 (CH-Ph), 126.2 (C4), 73.2 (OCH_2Ph), 67.7 (CH_2OBn), 54.9 (CH_2N). HRMS-ESI: m/z $[M+H]^+$ calcd for $C_{19}H_{19}N_2O_2$: 307.14410, found: 307.14329.

4.1.9. 2-Benzyl-4-benzyloxymethylpyridazin-3(2H)-one (**5d**)

Compound **5d** (12 mg, 56%), yellowish oil, was obtained from **5c** (15 mg, 0.07 mmol) following the same procedure as for preparation of compound **4b** from **4a** (Method D). R_f = 0.5 (hexane/ethyl acetate, 2:1). ^1H NMR ($CDCl_3$): δ = 7.79 (d, 1H, J = 4.0 Hz, H6), 7.44–7.40 (m, 2H, H-Ph), 7.37–7.27 (m, 9H, H5, H-Ph), 5.33 (s, 2H, CH_2N), 4.66 (s, 2H, OCH_2Ph), 4.54 (d, 2H, J = 1.7 Hz, CH_2OBn). ^{13}C NMR ($CDCl_3$): δ = 159.5 (C3), 141.0 (C4), 137.7 (C-Ph), 136.6 (C6), 136.3 (C-Ph), 128.9 (CH-Ph), 128.7 (CH-Ph), 128.7 (CH-Ph), 128.1 (CH-Ph), 128.1 (CH-Ph), 127.9 (CH-Ph), 126.0 (C5), 73.5 (OCH_2Ph), 66.6 (CH_2OBn), 55.2 (CH_2N). HRMS-ESI: m/z $[M+H]^+$ calcd for $C_{19}H_{19}N_2O_2$: 307.14410, found: 307.14494.

4.1.10. 2-Benzyl-5-bromomethylpyridazin-3(2H)-one (**4e**)

To a solution of compound **4c** (83 mg, 0.39 mmol) in CH_2Cl_2 (5 mL) was added CBr_4 (256 mg, 0.77 mmol), PPh_3 (202 mg, 0.77 mmol) and the reaction mixture was refluxed for 1.5 h. After quenching with $NaHCO_3$ (sat. aqueous solution, 5 mL) the product was extracted with CH_2Cl_2 (3 × 5 mL), dried over Na_2SO_4, and the solvent was removed under reduced pressure. The residue was purified by column chromatography on silica gel (dichloromethane/methanol, 99.5:0.5) to afford **4e** (62 mg, 58%) as a yellowish oil. R_f = 0.6 (ethyl acetate/methanol, 95:5). ^1H NMR ($CDCl_3$): δ = 7.77 (d, 1H, J = 1.9 Hz, H6), 7.44–7.39 (m, 2H, H-Ph), 7.35–7.27 (m, 3H, H-Ph), 6.85 (d, 1H, J = 1.9 Hz, H4), 5.30 (s, 2H, CH_2N), 4.17 (s, 2H, CH_2Br). ^{13}C NMR ($CDCl_3$): δ = 159.7 (C3), 141.7 (C5), 137.0 (C6), 136.1 (C-Ph), 128.9 (CH-Ph), 128.8 (CH-Ph), 128.2 (CH-Ph), 128.0 (C4), 55.2 (CH_2N), 27.0 (CH_2Br). HRMS-ESI: m/z $[M+H]^+$ calcd for $C_{12}H_{12}BrN_2O$: 279.01275, found: 279.01210.

4.1.11. 2-Benzyl-4-bromomethylpyridazin-3(2H)-one (**5e**)

Compound **5e** (31 mg, 84%), white solid, was obtained from **5c** (29 mg, 0.13 mmol) following the same procedure as for preparation of compound **4e** from **4c**. Compound **5e** was purified by column chromatography on silica gel (hexane/ethyl acetate, 6:1). R_f = 0.2 (hexane/ethyl acetate, 6:1). ^1H NMR ($CDCl_3$): δ = 7.77 (d, 1H, J = 4.0 Hz, H6), 7.46–7.41 (m, 2H, H-Ph), 7.36–7.27 (m, 4H, H5, H-Ph), 5.35 (s, 2H, CH_2N), 4.39 (s, 2H, CH_2Br). ^{13}C NMR ($CDCl_3$): δ = 159.5 (C3), 138.9 (C4), 136.1 (C6), 136.0 (C-Ph), 129.7 (C5), 129.0 (CH-Ph), 128.8 (CH-Ph), 128.2 (CH-Ph), 55.7 (CH_2N), 26.6 (CH_2Br). HRMS-ESI: m/z $[M+H]^+$ calcd for $C_{12}H_{12}BrN_2O$: 279.01275, found: 279.01337.

4.1.12. 4,4a,6,7,7a,1-Hexahydro-7a-hydroxyfuro[3,2-c]pyridazin-3(2H)-one (**14**)

To a solution of compound **12** [20] (30 mg, 0.19 mmol) in ethanol (4 mL) was added hydrazine monohydrate (37 μL, 0.76 mmol). The reaction mixture was refluxed for 5 h, and the solvent was removed obtaining **14** (29 mg, 97%) as a white solid. R_f = 0.1 (ethyl acetate/methanol, 95:5). ^1H NMR (CD_3OD): δ = 4.11 (d, 1H, J = 6.7 Hz, H4a), 3.99–3.92 (m, 1H, H6), 3.88–3.82 (m, 1H, H6), 2.76 (dd, 1H, J = 18.0, 6.7 Hz, H4), 2.45–2.38 (m, 1H, H7), 2.26 (d, 1H, J = 18.0 Hz, H4), 2.13–2.05 (m, 1H, H7). ^{13}C NMR (CD_3OD): δ = 173.2 (C3), 100.4 (C7a), 80.7 (C4a), 68.4 (C6), 37.1 (C7), 36.0 (C4).

4.1.13. 4,4a,6,7,8,8a-Hexahydro-1H-8a-hydroxypyrano[3,2-c]pyridazin-3(2H)-one (**15**)

Compound **15** (113 mg, 98%), white solid, was obtained from **13** [20] (115 mg, 0.67 mmol) following the same procedure as for preparation of compound **14** from **12**. R_f = 0.2 (ethyl acetate/methanol, 95:5). ^1H NMR (CD_3OD): δ = 3.85 (d, 1H, J = 4.8 Hz, H4a), 3.83–3.74 (m, 1H, H6), 3.46–3.36 (m, 1H, H6), 2.76 (dd, 1H, J = 16.9, 4.8 Hz, H4), 2.59–2.50 (m, 1H, H7), 2.09 (d, 1H, J = 16.9 Hz, H4), 1.74–1.63 (m, 1H, H7), 1.60–1.51 (m, 2H, H8). ^{13}C NMR (CD_3OD): δ = 176.5 (C3), 88.3 (C8a), 76.5 (C4a), 66.6 (C6), 38.6 (C4), 30.3 (C7), 23.2 (C8). HRMS-ESI: m/z $[M+H]^+$ calcd for $C_7H_{13}N_2O_3$ 173.09207, found: 173.09227.

4.1.14. 4,4a,6,7-Tetrahydrofuro[3,2-c]pyridazin-3(2H)-one (**6a**)

To a solution of compound **14** (33 mg, 0.21 mmol) in CH_2Cl_2 (4 mL) was added $BF_3{\cdot}OEt_2$ (31 μL, 0.25 mmol) at 0 °C. The reaction mixture was stirred at r.t. for 14 h, quenched with $NaHCO_3$ (sat. aqueous solution, 0.5 mL), dried over Na_2SO_4 and filtered. After removal of the solvent the residue was purified by column chromatography on silica gel (ethyl acetate) to afford **6a** (28 mg, 96%) as

a colorless oil. R_f = 0.5 (ethyl acetate/methanol, 95:5). ^{1}H NMR (CD_3OD): δ = 4.49–4.42 (m, 1H, H4a), 4.31–4.25 (m, 1H, H6), 4.08–4.01 (m, 1H, H6), 2.85–2.67 (m, 2H, H7), 2.81 (dd, 1H, J = 16.1, 7.7 Hz, H4), 2.50 (dd, 1H, J = 16.1, 13.9 Hz, H4). ^{13}C NMR (CD_3OD): δ = 168.8 (C3), 164.3 (C7a), 72.6 (C4a), 69.0 (C6), 34.5 (C4), 30.3 (C7). HRMS-EI: m/z $[M]^+$ calcd for $C_6H_8N_2O_2$, 140.0586, found: 140.0591.

4.1.15. 4,4a,7,8-Tetrahydro-6H-pyrano[3,2-c]pyridazin-3(2H)-one (**6b**)

To a solution of compound **15** (50 mg, 0.29 mmol) in CH_2Cl_2 (5 mL) was added $BF_3{\cdot}OEt_2$ (44 μL, 0.36 mmol) at 0 °C. The reaction mixture was stirred at 0 °C for 1.5 h, quenched with $NaHCO_3$ (sat. aqueous solution, 0.5 mL), dried over Na_2SO_4 and filtered. After removal of the solvent the residue was purified by column chromatography on silica gel (hexane/ethyl acetate, 7:3) to afford **6b** (33 mg, 73%) as a white solid. R_f = 0.4 (ethyl acetate/methanol, 95:5). ^{1}H NMR (CD_3OD): δ = 4.37–4.30 (m, 1H, H4a), 4.01–3.95 (m, 1H, H6), 3.65–3.57 (m, 1H, H6), 2.82 (dd, 1H, J = 17.4, 9.0 Hz, H4), 2.63–2.56 (m, 1H, H8), 2.53 (dd, 1H, J = 17.4, 11.5 Hz, H4), 2.51–2.41 (m, 1H, H8), 1.92–1.86 (m, 2H, H7). ^{13}C NMR (CD_3OD): δ = 168.7 (C3), 153.1 (C8a), 70.9 (C4a), 67.4 (C6), 34.5 (C4), 30.3 (C8), 26.9 (C7). HRMS-ESI: m/z $[M+H]^+$ calcd for $C_7H_{11}N_2O_2$, 155.08150, found: 155.08147.

4.1.16. 7,8-Dihydro-6H-pyrano[3,2-c]pyridazin-3(2H)-one (**6c**)

To a solution of compound **6b** (16 mg, 0.10 mmol) in DMF (3 mL) was added MnO_2 (169 mg, 1.94 mmol) and the reaction mixture was refluxed for 5 h. The mixture was filtered and the solvent was removed in vacuo. The residue was purified by column chromatography on silica gel (ethyl acetate) to afford **6c** (7 mg, 47%) as a white solid. R_f = 0.2 (ethyl acetate/methanol, 95:5). ^{1}H NMR (CD_3OD): δ = 6.12 (s, 1H, H4), 4.28 (t, 2H, J = 5.3 Hz, H6), 2.83 (t, 2H, J = 6.5 Hz, H8), 2.12–2.05 (m, 2H, H7). ^{13}C NMR (CD_3OD): δ = 166.4 (C3), 160.4 (C4a), 140.2 (C8a), 107.5 (C4), 69.1 (C6), 26.6 (C8), 22.6 (C7). HRMS-ESI: m/z $[M+H]^+$ calcd for $C_7H_9N_2O_2$, 153.06585, found: 153.06537.

4.2. Antiplatelet activity studies

Washed human platelets were prepared from blood anticoagulated with citrate–phosphate–dextrose, which was obtained from Centro de Transfusión de Galicia (Santiago de Compostela. Spain). Bags containing buffy coat from individual donors were diluted with the same volume of washing buffer (NaCl, 120 mM; KCl, 5 mM; trisodium citrate, 12 mM; glucose, 10 mM; sucrose, 12.5 mM; pH 6) and centrifuged at 400 g for 8 min. The upper layer containing platelets (platelet rich plasma) was removed and centrifuged at 1100 g for 18 min. The resulting platelet pellet was recovered, resuspended with washing buffer, and centrifuged again at 1100 g for 15 min. Finally, the platelet pellet from this step was resuspended in a modified Tyrode–HEPES buffer (HEPES, 10 mM; NaCl, 140 mM; KCl, 3 mM; $MgCl_2$, 0.5 mM; $NaHCO_3$, 5 mM; glucose, 10 mM; pH 7.4) to afford a cell density of 3–3.5 × 10^8 platelet/mL. The calcium concentration in the extracellular medium was adjusted to 2 mM by the addition of the appropriate amount of $CaCl_2$.

Platelet aggregation was measured using a dual channel aggregometer (Chrono-log, Havertown, PA, USA). Each tested compound, dissolved in DMSO, was incubated with washed platelets at 37 °C for 5 min. A platelet activator (either collagen or CRP or thrombin or U-46619 or ionomycin) was then added to induce platelet aggregation and the light transmission was monitored over a 5 min period. Platelet aggregation is measured as the maximum change in light transmission during this period. The 100% aggregation value was obtained when vehicle (DMSO) was added instead of the compounds. The final DMSO concentration was below 1% (v/v) in all cases.

Results shown in Table 1 are expressed as means ± SEM from at least four experiments.

4.2.1. Effect on Gp IIb/IIIa activation

Expression of activated GpIIb/IIIa on platelets membrane was analyzed using the specific FITC-labeled monoclonal antibody PAC-1, which recognizes an epitope on the activated form of glycoprotein IIb/IIIa complex of stimulated platelets.

Washed human platelets were pre-incubated with DMSO or test compounds for 5 min, and then stimulated with CRP-XL in the presence of FITC conjugated PAC-1 monoclonal antibody for 15 min at room temperature. The samples were then diluted and analyzed on a Beckman Coulter EPICS XL flow cytometer with System II software. Platelets were identified by logarithmic signal amplification for forward and side scatter. For every histogram, 10,000 platelets events were counted to evaluate both PAC-1 mean fluorescence intensity and the percentage of platelets positive for PAC-1 expression. All assays included samples to which RGDS peptide was added in order to determine the specificity of PAC-1 binding.

4.2.2. Effect on protein tyrosine phosphorylation

Washed platelet lysates were prepared by addition of SDS/EDTA (2%/1 mM) final concentration) to samples of platelets pre-incubated with DMSO (control) or tested compounds at 37 °C for 5 min and treated with or without (unstimulated samples) platelet aggregation inducers in the aggregometer as indicated above. The samples were later boiled for 5 min and the proteins contained in platelet lysates were transferred onto Polyvinylidene Fluoride (PVDF) membranes using a dot-blot filtration manifold. After blotting, the membranes were blocked overnight in 5% bovine serum albumin (BSA) in Tris-buffered saline supplemented with 0.1% Tween 20 (TBST), washed three times in TBST, and incubated for 1 h in the anti-phosphotyrosine 4G10 antibody solution prepared in TBST with 1% bovine serum albumin. After washing with TBST, the membrane was incubated with horseradish peroxidase-conjugated secondary antibody for 1 h. After washing with TBST, protein spots on the membrane were visualized by an enhanced chemiluminescence Western blotting detection system (Pierce, Thermo Scientific).

Acknowledgments

We acknowledge the Xunta de Galicia (CN2012/184) for the financial support. M.C.C.-L. and N.V. thank the Xunta de Galicia and the Universidade de Vigo for their PhD fellowships.

References

[1] C. Kennedy, Future Med. Chem. 5 (2013) 355–357.
[2] (a) A. Tello-Montoliu, E. Jover, J. Rivera, M. Valdés, D.J. Angiolillo, F. Marín, Curr. Med. Chem. 19 (2012) 406–427;
(b) T.-I. Siddiqui, K.S.A. Kumar, D.K. Dikshit, Curr. Med. Chem. 20 (2013) 2779–2797;
(c) K. Kolandaivelu, D.L. Bhatt, Novel antiplatelet therapies, in: A.D. Michelson (Ed.), Platelets, third ed., Elsevier, London, 2013, pp. 1185–1213.
[3] (a) J.A. Bristol, I. Sircar, W.H. Moos, D.B. Evans, R.E. Weishaar, J. Med. Chem. 27 (1984) 1099–1101;
(b) W.H. Moos, C.C. Humblet, I. Sircar, C. Rithner, R.E. Weishaar, J.A. Bristol, A.T. McPhail, J. Med. Chem. 30 (1987) 1963–1972;
(c) N.N. Smolyar, Y.M. Yutilov, S.V. Gres'ko, Pharm. Chem. J. 43 (2009) 87–88.
[4] (a) S. Demirayak, A.C. Karaburun, R. Beis, Eur. J. Med. Chem. 39 (2004) 1089–1095;
(b) K. Abouzid, M.A. Hakeem, O. Khalil, Y. Maklad, Bioorg. Med. Chem. 16 (2008) 382–389.
[5] (a) C. Schudt, S. Winder, B. Müller, D. Ukena, Biochem. Pharmacol. 42 (1991) 153–162;

(b) S. Corsano, R. Vezza, R. Scapicchi, S. Foresi, G. Strappaghetti, G.G. Nenci, P. Gresele, Eur. J. Med. Chem. 30 (1995) 627–631.
[6] M. Asif, Curr. Med. Chem. 19 (2012) 2984–2991.
[7] (a) R.E. Weishaar, M.H. Cain, J.A. Bristol, J. Med. Chem. 28 (1985) 537–545;
(b) W.J. Coates, H.D. Prain, M.L. Reeves, B.H. Warrington, J. Med. Chem. 33 (1990) 1735–1741.
[8] (a) L. Betti, M. Floridi, G. Giannaccini, F. Manetti, G. Strappaghetti, A. Tafi, M. Botta, Bioorg. Med. Chem. Lett. 13 (2003) 171–173;
(b) L. Betti, M. Zanelli, G. Giannaccini, F. Manetti, S. Schenone, G. Strappaghetti, Bioorg. Med. Chem. 14 (2006) 2828–2836.
[9] (a) A. Coelho, E. Raviña, N. Fraiz, M. Yáñez, R. Laguna, E. Cano, E. Sotelo, J. Med. Chem. 50 (2007) 6476–6484;
(b) C. Meyers, M. Yañez, A. Elmaatougi, T. Verhelst, A. Coelho, N. Fraiz, G.L.F. Lemière, X. García Mera, R. Laguna, E. Cano, B.U.W. Maes, E. Sotelo, Bioorg. Med. Chem. Lett. 18 (2008) 793–797.
[10] T. Costas, P. Besada, A. Piras, L. Acevedo, M. Yañez, F. Orallo, R. Laguna, C. Terán, Bioorg. Med. Chem. Lett. 20 (2010) 6624–6627.
[11] A.M. Montaña, P.M. Grima, M. Castellví, C. Batalla, M. Font-Bardia, Tetrahedron 68 (2012) 9982–9998.
[12] E. Bures, P.G. Spinazzé, G. Beese, I.R. Hunt, C. Rogers, B.A. Keay, J. Org. Chem. 62 (1997) 8741–8749.
[13] M.R. Kernan, D.J. Faulkner, J. Org. Chem. 53 (1988) 2773–2776.
[14] S.N. Patil, F. Liu, J. Org. Chem. 73 (2008) 4476–4483.
[15] M. Aquino, I. Bruno, R. Riccio, L. Gomez-Paloma, Org. Lett. 8 (2006) 4831–4834.
[16] M.C. Costas-Lago, T. Costas, N. Vila, C. Terán, Acta Cryst. E69 (2013) o1826–o1827.
[17] M.C. Costas-Lago, T. Costas, N. Vila, P. Besada, Acta Cryst. E69 (2013) o1859–o1860.
[18] P. Besada, T. Costas, N. Vila, C. Chessa, C. Terán, Magn. Reson. Chem. 49 (2011) 437–442.
[19] E. Sotelo, E. Raviña, I. Estevez, J. Heterocycl. Chem. 36 (1999) 985–990.
[20] M. Pérez, P. Canoa, G. Gómez, C. Terán, Y. Fall, Tetrahedron Lett. 45 (2004) 5207–5209.
[21] L. Estévez, P. Besada, Y. Fall, M. Teijeira, C. Terán, Synthesis (2010) 425–430.
[22] G.V.R. Born, Nature 194 (1962) 927–929.
[23] S. Vilar, E. Quezada, L. Santana, E. Uriarte, M. Yañez, N. Fraiz, C. Alcaide, E. Cano, F. Orallo, Bioorg. Med. Chem. Lett. 16 (2006) 257–261.
[24] K.J. Clemetson, J.M. Clemetson, Thromb. Haemost. 86 (2001) 189–197.
[25] T.M. Quinton, F. Ozdener, C. Dangelmaier, J.L. Daniel, S.P. Kunapuli, Blood 99 (2002) 3228–3234.
[26] B.T. Atkinson, M.J. Stafford, C.J. Pears, S.P. Watson, Eur. J. Biochem. 268 (2001) 5242–5248.
[27] S.J. Shattil, Thromb. Haemost. 82 (1999) 318–325.
[28] M.H. Ginsberg, X. Du, E.F. Plow, Curr. Opin. Cell. Biol. 4 (1992) 766–771.
[29] T.M. Leisner, J.D. Wencel-Drake, W. Wang, S.C. Lam, J. Biol. Chem. 274 (1999) 12945–12949.
[30] S.J. Shattil, J.A. Hoxie, M. Cunningham, L.F. Brass, J. Biol. Chem. 260 (1985) 11107–11114.
[31] B.S. Coller, J.D. Folts, S.R. Smith, L.E. Scudder, R. Jordan, Circulation 80 (1989) 1766–1774.
[32] H. Darius, Thromb. Res. 103 (2001) 117–124.
[33] M. Tsuji, Y. Ezumi, M. Arai, H. Takayama, J. Biol. Chem. 272 (1997) 23528–23531.
[34] J.M. Clemetson, J. Polgar, E. Magnenat, T.N. Wells, K.J. Clemetson, J. Biol. Chem. 274 (1999) 29019–29024.
[35] Y. Ezumi, K. Shindoh, M. Tsuji, H. Takayama, J. Exp. Med. 188 (1998) 267–276.
[36] S.P. Watson, J.M. Auger, O.J. McCarty, A.C. Pearce, J. Thromb. Haemost. 3 (2005) 1752–1762.