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RESEARCH PAPER

The sGC stimulator riociguat inhibits platelet function in washed platelets but not in whole blood

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BACKGROUND AND PURPOSE

Stimulation of soluble guanylyl cyclase (sGC) is a valuable therapeutic strategy for the treatment of several cardiovascular diseases. The sGC stimulator riociguat has been approved for the treatment of two forms of pulmonary hypertension. Platelets contain large amounts of sGC and play a key role in the regulation of haemostasis. Therefore, we investigated the effects of riociguat on platelet function.

EXPERIMENTAL APPROACH

The effect of riociguat treatment on human platelet activation and aggregation was investigated. The sGC-specific effects of riociguat were determined by comparing wild-type and platelet-specific sGC-knockout mice.

KEY RESULTS

Riociguat induced cGMP synthesis and subsequent PKG activation in human platelets, suggesting that the inhibitory effects are mediated by cGMP signalling. This finding was confirmed when sGC-knockout platelets were not inhibited by riociguat. In washed human platelets, 100 nM riociguat reduced ADP-induced GPIIb/IIIa activation, while a 10-fold higher concentration was required to reduce convulxin-stimulated GPIIb/IIIa activation. Riociguat inhibited ADP-induced platelet shape change and aggregation, while ATP-induced shape change remained unaffected. However, in PRP and whole blood, 50–100 μM riociguat was required to inhibit platelet activation and aggregation. Riociguat in combination with iloprost significantly inhibited platelet aggregation, even in whole blood.

CONCLUSIONS AND IMPLICATIONS

Riociguat inhibits platelet activation in whole blood only at concentrations above 50 μM, while the plasma concentrations in riociguat-treated patients are 150 to 500 nM. This finding indicates that riociguat treatment does not affect platelet function in patients. Nevertheless, the possibility that riociguat acts synergistically with iloprost to inhibit platelet activation should be considered.

Abbreviations

CTEPH, chronic thromboembolic pulmonary hypertension; FDA, Food and Drug Administration; LaSca, low-angle scattering; PAH, pulmonary arterial hypertension; sGC, soluble guanylyl cyclase

Tables of Links

TARGETS	
GPCRs[a]	**Enzymes**[d]
P2Y1 receptor	PDE3
P2Y12 receptor	PKA
Ligand-gated ion channels[b]	PKG
P2X1 receptor	sGC
Catalytic receptors[c]	
Integrins, GpIIb/IIIa	

LIGANDS	
ADP	MRS2179
ATP	NF449
cAMP	NO
cGMP	PAC-1
Iloprost	Riociguat

These Tables list key protein targets and ligands in this article which are hyperlinked to corresponding entries in http://www.guidetopharmacology.org, the common portal for data from the IUPHAR/BPS Guide to PHARMACOLOGY (Pawson *et al.*, 2014) and are permanently archived in the Concise Guide to PHARMACOLOGY 2013/14 ([a,b,c,d]Alexander *et al.*, 2013a,b,c,d).

Introduction

Platelets play a key role in normal and pathological haemostasis through their ability to adhere rapidly to activated or injured endothelium and subendothelial matrix proteins (platelet adhesion) and to other activated platelets (platelet aggregation). When the vessel wall is damaged, platelets are activated by subendothelial matrix proteins and von Willebrand factor released from endothelial cells. The activated platelets release secondary platelet-activating molecules, ADP and thromboxane A_2, to attract more platelets to the site of injury and finally form platelet aggregates to prevent bleeding (Ni and Freedman, 2003; Ruggeri and Mendolicchio, 2007; Varga-Szabo *et al.*, 2008). Platelets in circulation are maintained in the resting state by endothelium-derived prostacyclin (PGI_2) and NO. PGI_2 stimulates adenylate cyclase to synthesize cAMP, which activates PKA. NO binds to the haem domain of the soluble guanylyl cyclase (sGC) and thereby stimulates the synthesis of cGMP, which activates PKG. Both PKA and PKG phosphorylate several substrates, resulting in the inhibition of calcium release, granule secretion, cytoskeleton reorganization, integrin activation and platelet aggregation (reviewed by Smolenski, 2012; Walter and Gambaryan, 2009).

It has become apparent that many cardiovascular diseases are associated with a dysfunction of the NO/sGC/cGMP system (Evora *et al.*, 2012; Roe and Ren, 2012; Erdmann *et al.*, 2013). Therefore, agents that stimulate cGMP synthesis via sGC are important therapeutic options for the treatment of several cardiovascular diseases (Evgenov *et al.*, 2006).

The pharmacological activation of sGC has been intensively investigated (Follmann *et al.*, 2013). Many NO donors are known to induce nitrate tolerance (Munzel *et al.*, 2005), affect the function of other proteins (e.g. nitrosation) (Alencar *et al.*, 2003) or contribute to oxidative stress (Gladwin, 2006; Stasch *et al.*, 2006). To overcome these problems, novel classes of compounds that can stimulate sGC independently of NO were developed (Evgenov *et al.*, 2006). The first sGC stimulator identified YC-1 (Ko *et al.*, 1994) had a poor pharmacokinetic profile and low specificity (Stasch and Hobbs, 2009). The optimization of the structure of YC-1 led to the development of two distinct classes of molecules, namely, sGC activators and sGC stimulators (Follmann *et al.*, 2013).

Soluble guanylyl cyclase activators can activate the enzyme independent of the haem group (Schmidt *et al.*, 2009). They bind to the oxidised haem-free sGC, which is generated in many cardiovascular diseases, making them promising drugs for the treatment of various cardiovascular diseases (Lapp *et al.*, 2009; Gheorghiade *et al.*, 2012). sGC stimulators are haem-dependent and show a dual mode of action by sensitizing sGC to endogenous NO and directly stimulating sGC (Stasch and Hobbs, 2009). The sGC activator (Cinaciguat; Bay 58-2667) and stimulators (Bay 41-2272 and Bay 41-8543) identified were less successful due to low efficacy, low oral bioavailability (Mittendorf *et al.*, 2009) and unwanted side effects (Gheorghiade *et al.*, 2012). Riociguat (BAY 63-2521) is the first sGC stimulator that has made a successful transition from animal experiments to controlled clinical studies in human patients. Riociguat showed favourable drug metabolism and pharmacokinetic profiles (Rickert *et al.*, 2014). In different experimental models of pulmonary hypertension, riociguat had beneficial effects on pulmonary haemodynamics, right heart hypertrophy and remodelling of the pulmonary vasculature (Stasch and Evgenov, 2013). In phase III clinical trials, riociguat demonstrated efficacy in patients with pulmonary arterial hypertension (PAH) and, remarkably, also in patients with chronic thromboembolic pulmonary hypertension (CTEPH) (Ghofrani *et al.*, 2013a; Ghofrani *et al.*, 2013b). Recently, riociguat (Adempas®) was approved by the Food and Drug Administration (FDA) and European Medicines Agency for the treatment of these two forms of pulmonary hypertension: inoperable recurrent or persistent CTEPH and PAH (Conole and Scott, 2013). The potentially increased risk of pulmonary bleeding with riociguat in some patients is reflected as a warning in local prescribing information, and prescribers should regularly assess an individual patient's risk of pulmonary bleeding with riociguat treatment. However, Frey and colleges (2008) showed that riociguat did not induce an increased bleeding risk in healthy human volunteers, even in the presence of warfarin or aspirin (Stasch and Hobbs, 2009; Frey *et al.*, 2011b; Frey *et al.*, 2011a).

Nevertheless, additional investigations into the antiplatelet properties of riociguat are needed. In the current study, we investigated the effects of riociguat on platelet activation and aggregation in washed platelets, PRP and whole blood. We showed that 100 nM riociguat reduced ADP-induced integrin activation in washed human platelets. This concentration is pharmacologically relevant, because it has been reported that administration of 2.5 mg riociguat results in riociguat plasma concentrations between 150 and 500 nM. However, 100-fold higher concentrations of riociguat (50 μM) were needed to induce platelet inhibitory effects in whole blood. In addition, our results suggest that riociguat shows a synergistic effect with iloprost at inhibiting platelet aggregation at pharmacologically relevant concentrations. This suggests that riociguat itself does not affect platelet function in riociguat-treated patients. Nevertheless, the synergistic effect of riociguat and iloprost indicates that riociguat has potential as an antiplatelet drug when combined with iloprost.

Methods

Mice

Platelet-specific sGC knockout (PS-GCKO) mice were generated as previously described (Rukoyatkina *et al.*, 2011). All mice were housed according to the German Animal Welfare Act in a barrier facility (ZVTE, Zentrale Versuchstiereinrichtung, Universitätsmedizin Mainz) with a 12-h light–dark cycle. Mice were kept in European style Type II individually ventilated cages with a maximum of five mice per cage under conventional conditions. Mice were backcrossed on a C57Bl/6 background. The mice used in the experiments were 8-week-old male WT and PS-GCKO. A total number of 11 WT and 11 PS-GCKO mice was used in our experiments. All studies involving animals are reported in accordance with the ARRIVE guidelines for reporting experiments involving animals (Kilkenny *et al.*, 2010; McGrath *et al.*, 2010).

Human and mouse platelet preparation

Citrated blood was obtained from healthy volunteers after informed consent, according to our institutional guidelines and the Declaration of Helsinki. The healthy volunteers did not take any drugs for at least 14 days before the study. Studies using human platelets were approved by the ethics committee of the University Mainz (Study No. 837.302.12; 25.07.12). PRP and washed platelets were prepared as previously described (Gambaryan *et al.*, 2010).

Platelets from WT and PS-GCKO mice were isolated as described previously (Rukoyatkina *et al.*, 2011). All mouse experiments were conducted in accordance with the German legislation on the protection of animals and approved by the local animal care committee.

cAMP and cGMP measurements

Levels of cAMP and cGMP were evaluated using a cAMP Enzyme immunoassay (EIA) kit and cGMP EIA kit, respectively, following the manufacturers' instructions (Cayman Chemical, Hamburg, Germany).

Western blot analysis

Washed platelets were stimulated for 2 min with the indicated concentrations of riociguat and stopped by adding SDS gel loading buffer. For Western blotting, cell lysates were separated by SDS-PAGE, transferred to nitrocellulose membranes, and the membranes were incubated with appropriate primary antibodies (phospho-vasodilator-stimulated-phosphoprotein (VASP) Ser^{239} and actin) overnight at 4°C. For visualization of the signal, goat anti-rabbit or anti-mouse IgG conjugated with horseradish peroxidase were used as secondary antibodies, followed by electrochemiluminescence (ECL) detection. Blots were scanned using SilverFast software (LaserSoft Imaging AG, Kiel, Germany) and analysed densitometrically by NIH Image J software for uncalibrated optical density.

Flow cytometry analysis of platelet activation

Platelet-rich plasma was diluted to 2×10^7 platelets·mL^{-1} and pre-incubated with serial dilutions of riociguat for 10 min before 2.5 μM ADP or 5 ng·mL^{-1} convulxin stimulation for 2 min at 37°C. Platelets were labelled with FITC-conjugated GPIIb/IIIa and P-selectin antibodies (BD-Bioscience, Heidelberg, Germany). Stimulation was stopped by diluting the platelets (1:10) in PBS, and the result was then analysed using flow cytometry. The flow cytometry was performed on a BD FACS Canto II using CELLQuest software, version 3.1f (Becton Dickinson, Heidelberg, Germany).

Low-angle scattering aggregation

Platelet shape change and aggregation were analysed as previously described (Mindukshev *et al.*, 2012). Briefly, PRP was diluted in 6 mL of modified HEPES buffer (pH 7.4, osmolarity 302 mOsm, containing 140 mM NaCl, 10 mM HEPES, 10 mM $NaHCO_3$, 2 mM KCl, 1 mM $MgCl_2$, 2 mM $CaCl_2$ and 5.5 mM D-glucose) to a density of 1×10^7 platelets·mL^{-1}. After 2 min of establishing a constant basal signal, ADP was added at the indicated concentration to the platelet suspension. Riociguat was added 5 min before stimulating the platelets, and the recordings were performed for an additional 10 min.

Light transmission aggregometry

Platelet aggregation was measured using an Apact (LabiTec, Ahrensburg, Germany) aggregometer. PRP or washed platelets were diluted to 2×10^8 platelet·mL^{-1} in Tyrode's buffer (140 mM NaCl, 2.7 mM KCl, 0.42 mM NaH_2PO_4, 12 mM $NaHCO_3$, 5.5 mM glucose, 5 mM HEPES, pH 7.4). Serial dilutions of riociguat were added 15 min before stimulating the platelets. Platelet aggregation was induced with ADP (2 μM) or collagen (1.5 μg·mL^{-1}). Aggregation was measured with continuous stirring at 1000 rpm at 37°C for 10 min.

Impedance aggregometry

Platelet aggregation in whole blood was analysed by multiplate® analysis according to the manufacturer's recommendations (Roche, Mannheim, Germany). Briefly, 300 μL citrated whole blood and 300 μL saline were added to the test cell. After 3 min of incubation at 37°C, the samples were activated with ADP (12.5 μM) or collagen (1 μg·mL^{-1}). Serial dilutions of riociguat were added 15 min before the stimulation, and aggregation curves were recorded for 6 min. Platelet

aggregation was determined as the area under the curve of arbitrary units (AU*min).

Flow chamber experiments

Platelet adhesion on collagen under flow was analysed using the Bioflux 1000 flow chamber (IUL, Königswinter, Germany) as previously described (Conant *et al.*, 2009). Briefly, whole blood was pre-incubated with the indicated concentrations of riociguat (0–200 μM) for 30 min, and platelets were stained with 4 μM calcein AM for 15 min. Whole blood was perfused over collagen I-coated Bioflux microfluidic channels (100 $\mu g \cdot mL^{-1}$) at a shear rate of 5 $dyne \cdot cm^{-2}$ for 10 min. The channels were washed with PBS at 2 $dyne \cdot cm^{-2}$ for 5 min, and endpoint images of three randomly chosen segments of each channel were taken. Adhering platelets were visualized using an AxioObserver Z1 inverted microscope (10× per 0.3 numerical apperture objective) (Carl Zeiss Jena, Jena, Germany) equipped with a 120 W metal halide fluorescent lamp source, and a Retiga Exi AQUA Qimaging Retiga camera.

Data analysis

All experiments were performed at least three times, and the data are expressed as means ± SD. Differences between groups were analysed by ANOVA, and Student's *t*-test was used when appropriate.

Materials

ADP, collagen and SNP were obtained from Sigma-Aldrich (Seelze, Germany). Riociguat (Bay 63-2521) was a kind gift from Bayer (Wuppertal, Germany). Iloprost (Bayer), NF449 (Sigma-Aldrich), AR-C69931 (The Medicines Company, Parsippany, NJ, USA), MRS2179 (Viozol, Eching, Germany) and calcein acetoxymethyl (AM) ester (Live Technologies, Darmstadt, Germany) were purchased. FITC conjugated anti-CD62P, and anti-GPIIb/IIIa antibodies were purchased from Becton Dickinson (Heidelberg, Germany), anti-phospho-V ASPSer^{239} was from Nanotools (Teningen, Germany) and polyclonal rabbit anti-actin antibody was from Santa Cruz Biotechnology (Heidelberg, Germany). Horseradish peroxidase conjugated goat anti-rabbit and anti-mouse antibodies were from Bio-Rad Laboratories, Inc. (Munich, Germany).

Results

Riociguat specifically stimulates sGC in platelets

Riociguat increases the cGMP level in the plasma of healthy volunteers (Frey *et al.*, 2008). Platelets are reported to secrete cyclic nucleotides via the multidrug resistance protein 4 transporter (Jedlitschky *et al.*, 2004). Therefore, we tested whether riociguat can stimulate cGMP synthesis in platelets. Riociguat increased cGMP levels and VASP Ser^{239} phosphorylation concentration-dependently in wild type (WT) but not in sGC knockout mouse platelets (Figure 1A). This finding demonstrates that riociguat stimulates cGMP synthesis specifically via the stimulation of sGC.

Similar to mouse platelets, Riociguat treatment concentration- and time-dependently increased cGMP levels in washed

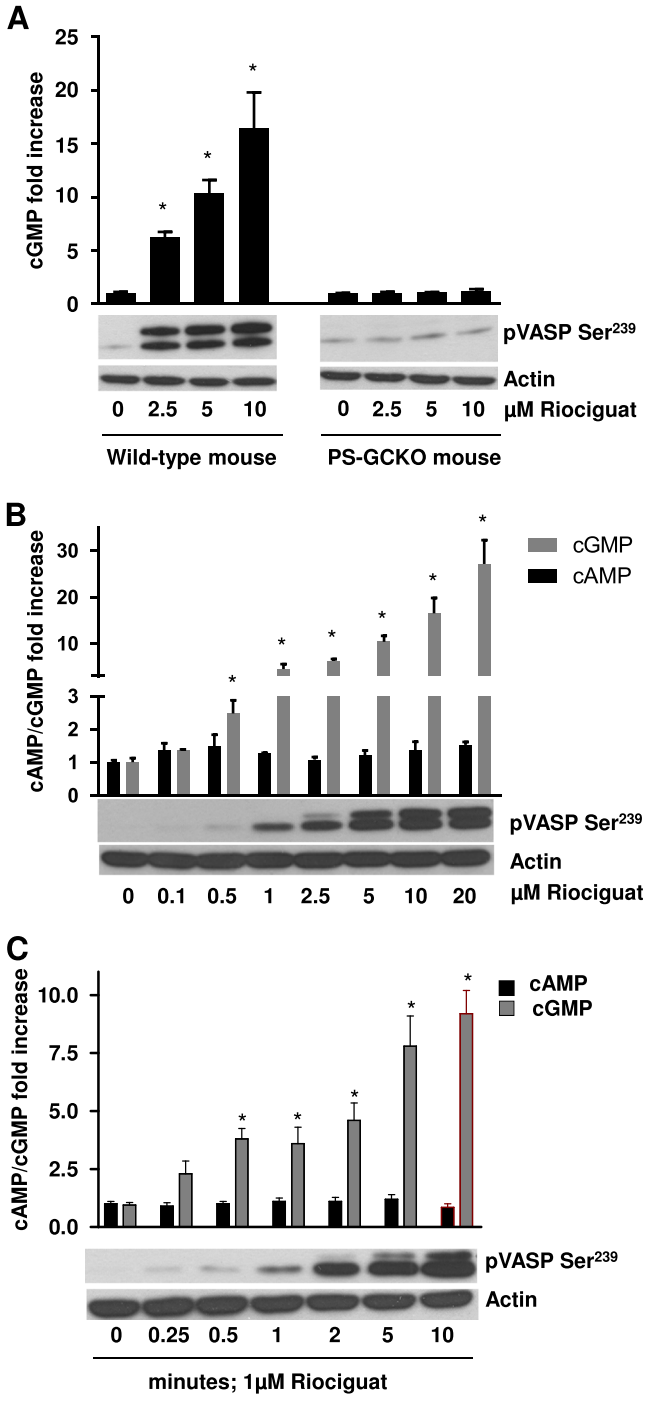

Figure 1

Riociguat stimulates sGC in washed platelets. (A) WT and PS-GCKO mouse platelets were stimulated with riociguat for 2 min. (B) Washed human platelets were stimulated with the indicated concentrations of riociguat for 2 min. (C) Washed human platelets were stimulated with 1 μM riociguat for the indicated time. cGMP and cAMP were quantified by EIA kits, and the phosphorylation of VASP Ser^{239} was detected by Western blot. Representative blots of three independent experiments are shown. Data are expressed as fold increase over unstimulated control and represent mean ± SD (n = 3). *$P < 0.05$ compared with control.

human platelets without affecting cAMP levels (Figure 1B and C). The basal cGMP concentration of 1.106 ± 0.324 pmol per 10^9 platelets was increased to 5.986 ± 0.17 pmol per 10^9 platelets by riociguat (1 μM) treatment, while the established NO-donor (SNP, 1 μM) increased the cGMP concentration to 8.439 ± 4.379 pmol per 10^9 platelets (Supporting Information Fig. 1A). The riociguat-induced cGMP increase led to PKG activation, as detected by the phosphorylation of VASP Ser^{239}. Riociguat-induced cGMP increase led to VASP phosphorylation in PRP as well as in whole blood (Supporting Information Fig. 1B). While 50 μM riociguat significantly increased VASP phosphorylation in PRP after 15 min treatment, this concentration had no effect on VASP phosphorylation in whole blood. A riociguat concentration of 200 μM was required to observe significant platelet VASP phosphorylation in whole blood after 15 min treatment, while 100 μM riociguat treatment for 30 min was sufficient to induce significant VASP phosphorylation (Supporting Information Fig. 1C). We therefore analysed the effects of riociguat on platelet functions after 15 and 30 min of riociguat treatment in PRP and whole blood respectively.

Riociguat inhibits agonist induced GPIIb/IIIa activation and α-granule secretion

We analysed whether the stimulation of sGC by riociguat can inhibit agonist-induced platelet activation. In washed human platelets, convulxin-induced GPIIb/IIIa activation (PAC-1 binding; Figure 2A) and α-granule secretion (P-selectin expression; Figure 2B) were significantly inhibited by ≥1 μM riociguat, whereas much lower concentrations of riociguat (>100 nM) were sufficient to significantly inhibit GPIIb/IIIa activation by ADP, a weak platelet agonist (Figure 2C). These data indicate that riociguat can inhibit platelet activation, and the concentration of riociguat required for this inhibitory effect depends on the agonist.

Riociguat inhibits ADP-induced platelet shape change and aggregation

It is well documented that activation of sGC/cGMP/PKG strongly inhibits platelet activation (Smolenski, 2012). However, the role of sGC activation in the inhibition of platelet shape change is not clear. Activation of sGC/PKG by SNP slightly increases cAMP concentrations, which activates PKA due to the inhibition of PDE3 (Dickinson *et al.*, 1997). Hence, it is challenging to determine the roles of cAMP and cGMP-mediated pathways in the inhibition of platelet shape change. It has previously been shown that platelet shape change is inhibited by PKA and not by PKG (Jensen *et al.*, 2004) when platelets were stimulated with cAMP or cGMP analogues. In the present study we demonstrated that riociguat, unlike SNP, does not increase cAMP concentrations, even at concentrations of 20 μM (Figure 1A). We, therefore, tested whether the specific effect of riociguat on sGC affects platelet shape change and aggregation by low-angle scattering (LaSca), which can detect shape change and aggregation in parallel (Mindukshev *et al.*, 2012). In human platelets, we found that shape change induced by 90 nM ADP was significantly reduced by riociguat, and riociguat completely inhibited platelet shape change induced by 40 nM ADP (Figure 3A, upper panel). Also, riociguat concentration-dependently reduced platelet aggregation induced by all the ADP concentrations tested (Figure 3A, lower panel).

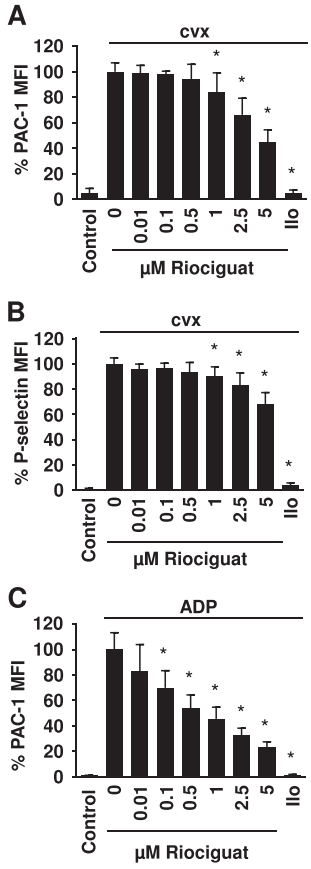

Figure 2

Riociguat inhibits platelet activation. PRP was diluted in HEPES buffer to a platelet count of 2 × 10^8 platelets·mL^{-1} and pre-incubated with either the indicated concentrations of riociguat for 10 min or iloprost (2 nM, 2 min) before stimulation with convulxin (cvx; 5 ng mL^{-1}, 2 min) or ADP (2.5 μM, 2 min). (A, C) PAC-1 binding and (B) P-selectin expression were determined by flow cytometry. Data are expressed as % of mean fluorescence intensities (MFI) of PAC-1 or P-selectin. The convulxin/ADP-stimulated sample was considered to be 100%. Data are presented as mean ± SD (n = 6). *P < 0.05 versus convulxin/ADP-stimulated sample.

Similar to human platelets, riociguat completely inhibited ADP-induced shape change and aggregation of WT mouse platelets but did not affect shape change and aggregation in sGC-deficient platelets stimulated with ADP, indicating that the inhibitory effect is specifically sGC-dependent (Figure 3B).

Next, we calculated the EC_{50} concentration of ADP required for shape change and aggregation in the presence of increasing concentrations of riociguat. We therefore plotted the amplitude of the shape change (I_{sh}) (Figure 3C) and the initial velocity of aggregation (U_{ia}) (Figure 3D) against the increased ADP concentrations and calculated the EC_{50} values as previously described (Mindukshev *et al.*, 2012). In our experiments, the EC_{50} of ADP for platelet shape change was 44 ± 6 nM (h = 1.25 ± 0.21), and pretreatment with riociguat increased the EC_{50} values of ADP for shape change in a concentration-dependent manner (Table 1). For platelet

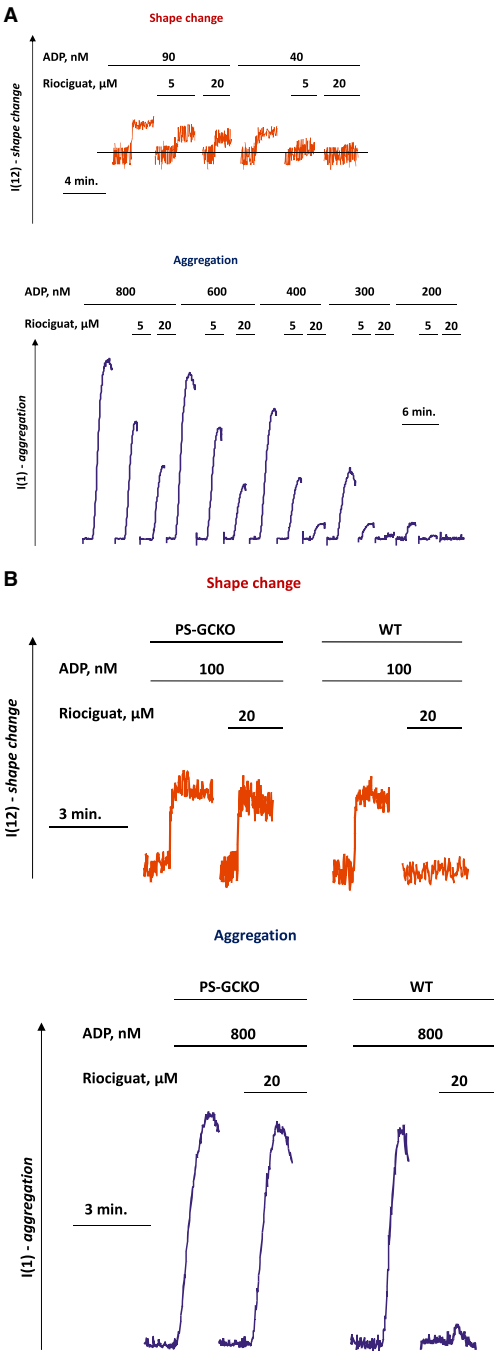

Figure 3

Riociguat inhibits platelet shape change and aggregation. (A) PRP was diluted in HEPES buffer to a platelet count of 1 × 10^7 platelets·mL^{-1} and pretreated with riociguat (2 min) before stimulation with the indicated concentrations of ADP. Platelet shape change and aggregation were determined using LaSca. Representative curves of shape change (upper panel) and aggregation (lower panel) are shown. (B) Diluted murine wild-type and PS-GCKO PRP were pre-incubated with riociguat (2 min) before ADP stimulation. Representative curves of shape change (upper panel) and aggregation (lower panel) are shown. (C, D) Diluted human PRP was stimulated as before, and the dose–response curves were plotted for (C) shape change and (D) aggregation. Data are presented as mean ± SD, $n = 4$.

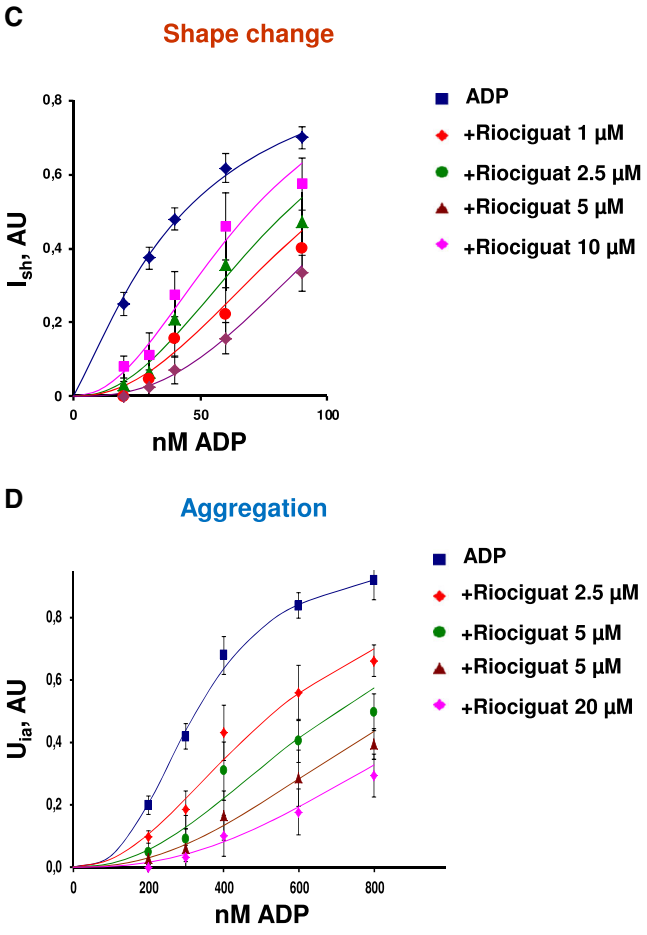

Figure 3

Continued

aggregation, the EC_{50} of ADP was 327 ± 22 nM (h = 2.78 ± 0.24). Similar to the results obtained for shape change, pretreatment with riociguat increased the EC_{50} values of ADP for aggregation (Table 1).

Next, we calculated the IC_{50} and binding affinity (Ki values) of riociguat for shape change and aggregation (Table 2 and Supporting Information). The dose–response curves revealed similar Ki values (~1 µM) for riociguat for shape change and aggregation. The IC_{50} concentrations of riociguat for shape change and aggregation were 6.8 and 10.4 µM respectively. For murine platelets, the calculations revealed similar Ki values of riociguat for shape change and aggregation (~1 µM), while the corresponding IC_{50} values were 10 and 7.9 µM respectively (Table 2).

Riociguat inhibits both P2Y1 and P2Y12 receptor-mediated platelet aggregation

The purine receptors, $P2Y_1$ and $P2Y_{12}$, mediate ADP-induced platelet activation and aggregation. In mice, it has been shown that signalling via both receptors is necessary for ADP-induced platelet aggregation (Murugappa and Kunapuli, 2006). To dissect the inhibitory potential of riociguat on $P2Y_1$- and $P2Y_{12}$ receptor-mediated signalling, platelets were pretreated with the $P2Y_1$ (MRS2179) or $P2Y_{12}$ (AR-C69931)

Table 1

Quantitative characterisation of the inhibitory effects of riociguat on ADP-induced platelet aggregation and shape change

	Shape change			Aggregation		
Stimulation	**EC_{50}, nM**	**n_H**	***n***	**EC_{50}, nM**	**n_H**	***n***
ADP	44 ± 6	1.25 ± 0.21	16	327 ± 22	2.78 ± 0.24	16
+ Riociguat 1 μM	70 ± 14	2.1 ± 0.29	4	538 ± 37	2.15 ± 0.29	4
+ Riociguat 2.5 μM	82 ± 17	2.2 ± 0.31	4	699 ± 41	2.26 ± 0.32	4
+ Riociguat 5 μM	99 ± 16	2.2 ± 0.29	4	894 ± 57	2.23 ± 0.29	4
+ Riociguat 10 μM	113 ± 19	2.62 ± 0.39	4	1070 ± 75	2.46 ± 0.37	4

The EC_{50} values for ADP to induce platelet shape change and aggregation in the presence of riociguat were calculated according to the experimental data presented in Figure 3C and D. n_H, Hill coefficient.

Table 2

Estimation of Ki from IC_{50} for riociguat of ADP-induced human and mouse platelet shape change and aggregation

Riociguat	**Shape change**		**Aggregation**	
	Human	**Mouse**	**Human**	**Mouse**
Ki, μM	0.96 ± 0.15	1.16 ± 0.1	0.91 ± 0.08	1.08 ± 0.18
IC_{50}, μM	6.8 (ADP = 90 nM)	10 (ADP = 90 nM)	10.4 (ADP = 800 nM)	7.9 (ADP = 800 nM)
n_H	2.5	2.5	2.4	2.3

Calculated values of Ki, IC_{50} and Hill coefficient (n_H) for ADP-induced platelet shape change and aggregation were calculated according to the experimental data presented in Figure 3. Further information is provided in the Supporting Information.

receptor antagonists before ADP stimulation. Pre-incubation with either MRS2179 or AR-C69931 markedly reduced ADP-induced aggregation by 60–75%, while either antagonist plus riociguat almost completely inhibited platelet aggregation (Figure 4). These data indicate that riociguat inhibits both $P2Y_1$ and $P2Y_{12}$ receptor-mediated platelet aggregation.

Riociguat inhibits $P2Y_1$ but not P2X1 receptor-mediated platelet shape change

Platelet shape change is mainly mediated via $P2Y_1$ receptors and P2X1, the receptor for ATP, on the platelet surface (Kahner *et al.*, 2006). In human platelets stimulated with 90 nM of ADP, 100 nM MRS2179 or 5 μM riociguat partially inhibited (35%) shape change. In platelets pretreated with both riociguat and MRS2179, we observed almost complete inhibition of ADP-stimulated platelet shape change, indicating that riociguat inhibits $P2Y_1$-mediated platelet shape change (Figure 5A). To rule out an effect of riociguat on P2X1 receptor-mediated shape change, platelets were pre-incubated with the same concentration of riociguat and then stimulated with ATP. However, neither SNP nor riociguat (even at 20 μM) inhibited ATP-induced platelet shape change, while the P2X1 antagonist (NF449) completely blocked shape change (Figure 5B). These results indicate that riociguat inhibits $P2Y_1$ but not P2X1 receptor-mediated platelet shape change.

Riociguat inhibits platelet aggregation in PRP and whole blood at concentrations above 50 μM

Next, we analysed whether the inhibitory effect of riociguat on platelet aggregation could be achieved in PRP and whole blood. In contrast to washed platelets, concentrations of >50 μM riociguat were required to inhibit ADP (Figure 6A), collagen (Figure 6B) and thrombin receptor activator peptide (TRAP)-6-induced aggregation (Figure 6C) in PRP and whole blood, probably due to the binding of riociguat to plasma proteins. No significant differences could be detected in the inhibitory effect of riociguat on ADP-induced aggregation between PRP and whole blood (Figure 6A). However, for collagen- and TRAP-6-induced aggregation, pretreatment with 200 μM riociguat completely abolished aggregation in PRP, while the same concentration of riociguat inhibited only 20% of aggregation in whole blood (Figure 6B and C), indicating that riociguat, in addition to plasma proteins, can bind to other blood cells.

Riociguat inhibits platelet adhesion under flow conditions

To test the platelet inhibitory potential of riociguat under more physiological conditions, Riociguat-pretreated whole blood was passed over collagen-coated channels, and platelet adhesion was analysed. We found that ≥100 μM riociguat was required to significantly inhibit platelet adhesion under flow

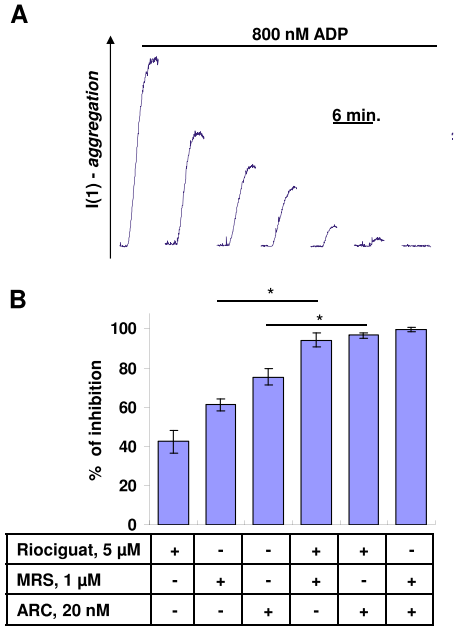

Figure 4

Riociguat inhibits both $P2Y_1$ and $P2Y_{12}$ receptor-mediated platelet aggregation. PRP was diluted in HEPES buffer to a platelet count of 1×10^7 platelets·mL^{-1} and pre-incubated with riociguat (5 µM, 2 min), MRS 2179, P2Y1 receptor antagonist (MRS, 1 µM, 2 min) AR-C69931, P2Y12 receptor antagonist (cangrelor, ARC 20 nM, 2 min) as indicated before ADP (800 nM) stimulation. (A) Platelet aggregation was monitored by LaSca. Representative aggregative curves are shown. (B) The graph represents % inhibition calculated from four independent experiments. Data are presented as mean ± SD. *$P < 0.05$ was considered significant.

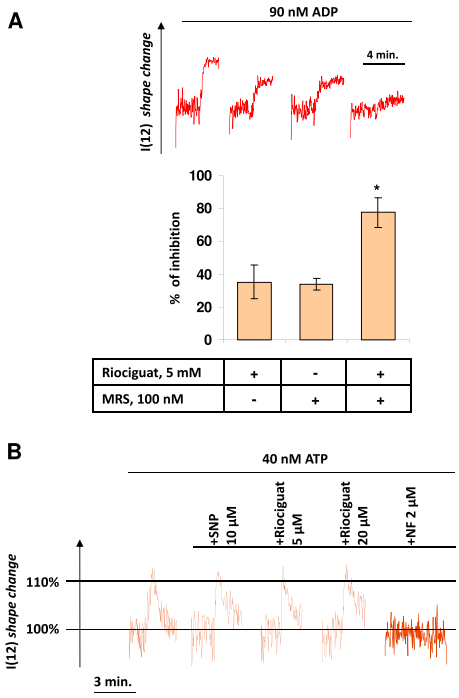

Figure 5

Riociguat inhibits $P2Y_1$ receptor-mediated but not P2X1 receptor-mediated platelet shape change. (A) PRP was diluted in HEPES buffer to a platelet count of 1×10^7 platelets·mL^{-1} and pre-incubated with MRS2179 (MRS, 100 nM, 2 min) followed by riociguat (5 µM, 2 min.) treatment. Platelets were stimulated with ADP, and shape change was monitored by LaSca. Representative shape change curves are shown (upper panel), and the graph (lower panel) represents % inhibition calculated from four independent experiments. Data are presented as mean ± SD. *$P < 0.05$ versus MRS treatment. (B) Diluted human PRP was pre-incubated with the indicated concentrations of SNP, riociguat or NF449 for 2 min before ADP stimulation. Representative shape change curves are shown. *$P < 0.05$ compared with MRS-treated sample.

conditions, whereas iloprost (2 nM) completely inhibited platelet adhesion (Figure 7).

Synergistic inhibitory effects of riociguat and iloprost on platelets

We investigated whether riociguat can intensify the platelet inhibitory potential of prostanoids, which activate cAMP-mediated signalling and are used for the treatment of pulmonary hypertension (Hoeper *et al.*, 2010). Combinations of riociguat and iloprost synergistically inhibited collagen-induced integrin GPIIb/IIIa activation (Figure 8A), α-granule secretion (Figure 8B) and ADP-induced integrin activation (Figure 8C), while the same concentrations of riociguat or iloprost alone had minimal effects in diluted PRP. Also, we found that riociguat acts together with iloprost at inhibiting ADP-induced platelet aggregation in whole blood (Figure 8D). These data indicate that riociguat and iloprost act synergistically to inhibit platelet activation in both PRP and whole blood.

Discussion

We present the first comprehensive study on the effects of riociguat on platelet activation. Riociguat was previously approved for the treatment of two forms of pulmonary hypertension, PAH and CTEPH (Conole and Scott, 2013). The FDA classified an increased risk of bleeding as a side effect of riociguat. In the present study, we evaluated whether riociguat affects platelet function.

Pulmonary arterial hypertension and CTEPH patients receive up to 2.5 mg riociguat per dose (three doses per day) (Ghofrani *et al.*, 2013a; Ghofrani *et al.*, 2013b). The pharmacokinetics of riociguat (Frey *et al.*, 2008; Grimminger *et al.*, 2009; Frey *et al.*, 2011a) showed that administration of 2.5 mg riociguat results in total plasma concentrations of 150–500 nM. In washed platelets, 500 nM riociguat increases cGMP concentrations threefold (Figure 1). The same concentration significantly reduces integrin activation induced by ADP, while a 10-fold higher concentration of riociguat was needed to reduce collagen-induced integrin activation and α-granule secretion (Figure 2).While 500 nM riociguat was sufficient to inhibit ADP-induced platelet activation, 5 µM was required to significantly reduce ADP-induced platelet aggregation in diluted PRP (Figure 3). We identified that both $P2Y_1$ and $P2Y_{12}$ receptor-mediated platelet aggregation was inhibited by the sGC/cGMP/PKG pathway (Figure 4).

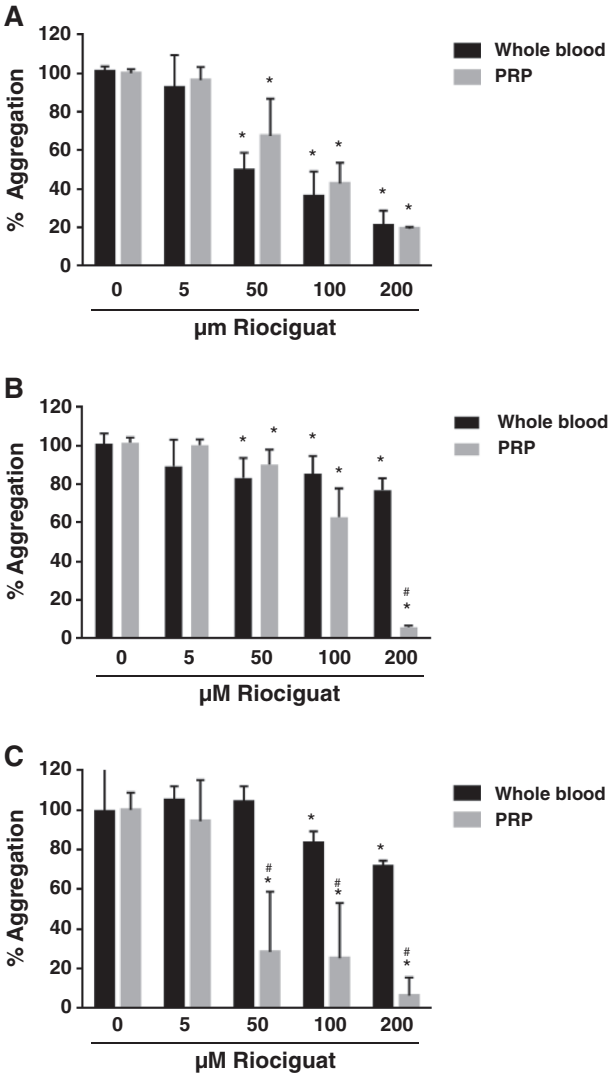

Figure 6

A high concentration of riociguat is required to inhibit platelet aggregation in PRP and whole blood. Whole blood or PRP was pre-incubated with the indicated concentrations of riociguat for 15 min before (A) ADP (2 µM for PRP; 12.5 µM for whole blood), (B) collagen (1.5 µg·mL^{-1} for PRP; 1 µg·mL^{-1} for whole blood) or (C) TRAP-6 (15 µM for PRP; 30 µM for whole blood) stimulation. Aggregation was measured using the light-transmission aggregometry for PRP and multiplate technology for whole blood. Data are expressed as % aggregation, and ADP/collagen-induced aggregation was considered to be 100%. Data represent mean + SD (n = 5). *P < 0.05, compared to without riociguat treatment; $^{\#}P$ < 0.05 versus corresponding whole blood.

Frey and colleges (2008) found that riociguat treatment had no effect on the bleeding time in healthy human volunteers. Furthermore, riociguat together with aspirin did not prolong the bleeding time compared with aspirin alone (Frey *et al.*, 2011b). In accordance with these observations, we showed that the inhibition of the platelet aggregation by riociguat was markedly reduced in PRP and whole blood (Figure 6). To obtain comparable data on platelet aggregation induced by different stimuli (ADP, collagen and TRAP-6) in

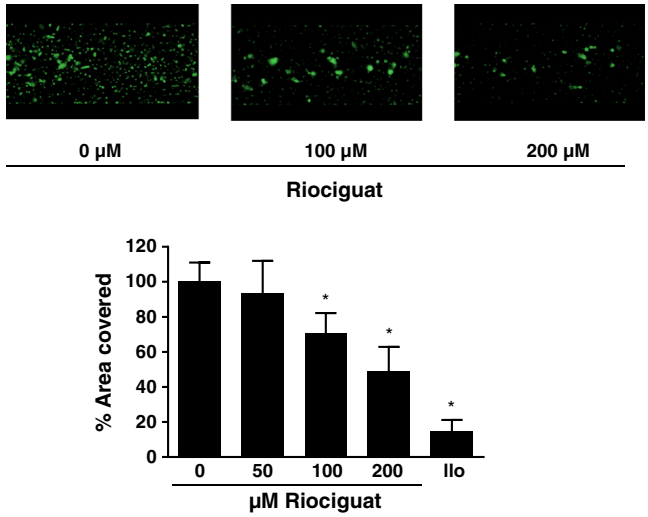

Figure 7

Inhibition of platelet adhesion by riociguat under flow conditions. Whole blood was pre-incubated with the indicated concentrations of riociguat for 30 min, and platelets were stained with calcein AM as mentioned in the methods. After the perfusion of whole blood over collagen-coated channels at a shear rate of 5 dyne·cm^{-2} for 10 min, end-point images of three randomly chosen segments of three independent channels were acquired by a fluorescence microscope at 10× magnification. Representative images for each sample (upper panel) are shown. Platelet adhesion was quantified as % of area covered using Bioflux montage software. The graph (lower panel) represents data from three independent experiments. Samples without riociguat treatment were considered to be 100%. Data are presented as mean ± SD. *P < 0.05 versus samples without riociguat treatment.

PRP and in whole blood, we determined the concentration for each agonist required to induce approximately 80% aggregation in preliminary experiments. For ADP-induced platelet aggregation (2 µM ADP in PRP and 12.5 µM in whole blood), concentrations of 50 µM riociguat were needed to induce a significant inhibition of platelet aggregation in PRP and whole blood (Figure 6A). Although we did not detect any differences in the inhibitory potential of riociguat on ADP-induced platelet aggregation in PRP and whole blood, significant differences were observed with collagen (1.5 µg mL^{-1} collagen in PRP and 1 µg mL^{-1} in whole blood) and TRAP-6-induced (15 µM TRAP-6 in PRP and 30 µM in whole blood) platelet aggregation (Figure 6B and C). In this study, 200 µM riociguat completely abolished platelet aggregation in PRP, whereas it only inhibited 20% of platelet aggregation in whole blood. This indicates that riociguat is scavenged by erythrocytes or other cells in whole blood, which is supported by the finding, that 200 µM riociguat strongly induces VASP Ser^{239} phosphorylation in PRP (20-fold over control) and only moderately induces VASP phosphorylation in whole blood (fivefold over control) (Supporting Information Fig. 1B). As ADP is a weak agonist, lower Riociguat concentrations are required to inhibit platelets, and this scavenging effect cannot be seen in whole blood.

Riociguat also inhibits platelet adhesion under flow in whole blood, but even higher concentrations of riociguat (100 µM) are required for this effect (Figure 7).

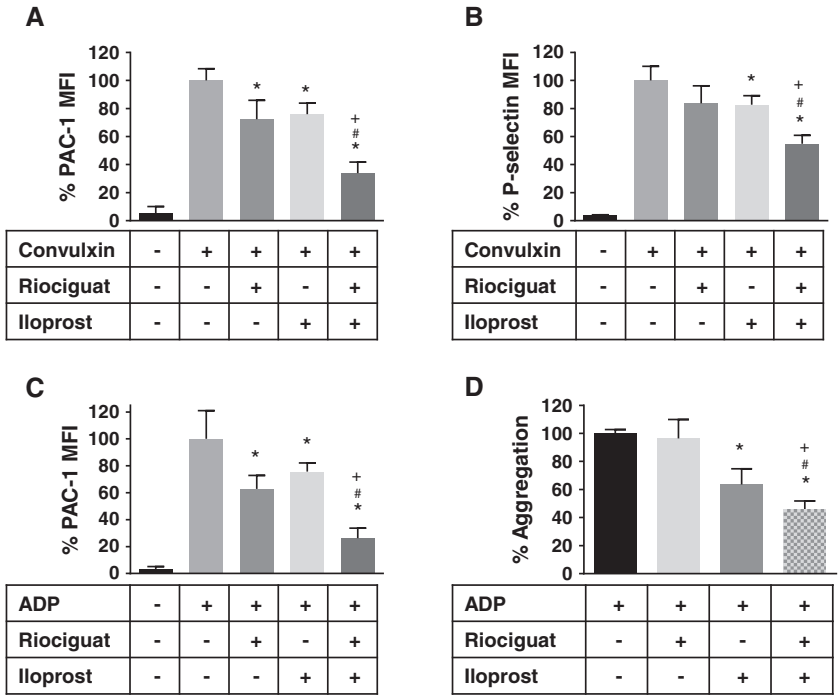

Figure 8

Synergistic inhibitory effects of riociguat and iloprost on platelets. (A, B) Diluted PRP was pre-incubated with riociguat (2 μM, 10 min) and iloprost (6 pM, 2 min) followed by convulxin (5 ng·mL^{-1}, 2 min) stimulation. (A) PAC-1 binding and (B) P-selectin expression were determined as described previously. (C) Diluted PRP was pre-incubated with riociguat (0.2 μM, 10 min) and iloprost (16 pM, 2 min) followed by ADP (2.5 μM, 2 min) stimulation. PAC-1 binding was measured. (D) Whole blood was pre-incubated with riociguat (500 nM, 15 min) and iloprost (500 nM, 2 min) followed by ADP (12.5 μM) stimulation. Aggregation was analysed using multiplate technology. (A–D) Data are presented as mean ± SD. Experiments were carried out at least three times and the ADP/convulxin-induced sample was considered to be 100%. *$P > 0.05$, compared to the convulxin-stimulated or ADP-stimulated sample; $^{\#}P < 0.05$ versus pre-incubation with iloprost alone; $^{+}P < 0.05$ versus pre-incubation with riociguat alone. MFI, mean fluorescence intensities.

Overall, riociguat affects platelet function in whole blood only at concentrations >50 μM, suggesting that riociguat is unlikely to increase the risk of bleeding in patients, where riociguat plasma concentrations of 150–500 nM are reported (Frey *et al.*, 2008). This hypothesis is supported by a pilot study in which riociguat was found to have not inhibitory effects on platelets *ex vivo*. We analysed VASP Ser^{239} phosphorylation and the platelet functions of integrin activation and P-selectin expression of one healthy volunteer 0, 3 and 24 h after administration of 2.5 mg riociguat and could not detect any differences (data not shown). The observation that the inhibitory effects of riociguat on platelets are reduced in plasma and whole blood is supported by previous studies. The sGC stimulator Bay 41-8543 showed increased IC_{50} values for collagen-induced aggregation in PRP when compared with washed platelets (Stasch *et al.*, 2002), and the sGC stimulator Bay 41-2272 showed a reduced potency in PRP, which was proposed to be partially due to binding to plasma proteins (Hobbs and Moncada, 2003). Our data taken together with previous findings indicate that platelet functions are not affected by riociguat treatment in patients.

A synergistic inhibitory effect of cAMP-dependent and cGMP-dependent pathways on platelets was observed when washed platelets or whole blood were simultaneously activated with concentrations of iloprost and riociguat (Figure 8), which do not show platelet-inhibitory effects when administered alone. Our data are supported by the finding that Bay 41-2272 has a synergistic effect with PGI_2, which leads to platelet inhibition. This synergistic effect was thought to be due to the cGMP-dependent inhibition of phosphodiesterase 3 (Hobbs and Moncada, 2003). However, we did not detect an increase in cAMP in platelets incubated with riociguat alone, indicating that this effect is mediated by increased cAMP levels induced by iloprost-mediated activation of adenylate cyclase. The clinical relevance of the synergistic effects of riociguat in combination with iloprost on platelet function is not known. However, riociguat has been shown to significantly improved exercise capacity, pulmonary vascular resistance and several other secondary efficacy end points in patients with symptomatic PAH who were receiving ilomedin (Ghofrani *et al.*, 2013b).

Another important question concerns platelet shape change, which was shown to be inhibited by PKA but not by PKG (Jensen *et al.*, 2004). In their study, the authors used cyclic nucleotide analogues, which are thought to specifically inhibit PKA and PKG, to prove their findings. However, we previously reported (Gambaryan *et al.*, 2004) that PKA and PKG inhibitors show nonspecific effects on platelet function. Here, we showed that riociguat does not increase cAMP concentrations (Figure 1B), and therefore, our data clearly show that ADP-induced platelet shape change can be inhibited by PKG without PKA activation. In addition, we showed that

riociguat inhibits $P2Y_1$ receptor-mediated shape change, while P2X1 receptor-mediated shape change was unaffected (Figure 5).

Taken together, our findings demonstrate that stimulation of platelet sGC by riociguat inhibits platelet activation and aggregation only at high pharmacological, therapeutically irrelevant drug concentrations. We found that the concentration of riociguat required to inhibit platelet functions in whole blood was 100-fold higher (50 μM) than that reached *in vivo* at therapeutically relevant doses (~500 nM). Hence, it is unlikely that riociguat, when used therapeutically, will inhibit platelet function, leading to enhanced bleeding risk in patients. Nevertheless, riociguat may act synergistically with iloprost or other prostanoids that increase cAMP levels when both drugs are used in combination; therefore, the possible inhibition of platelet functions must be considered when both drugs are administered concomitantly. Furthermore, this synergistic effect of riociguat and iloprost suggests that riociguat has potential as an antiplatelet drug.

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Author contributions

U. W., S. G. and C. R. participated in the research design. C. R., I. M., V. B. and L. K. conducted the experiments. J.-P. S. and A. F. contributed new reagents or mice. I. M. and C. R. performed the data analysis. C. R., H. S., S. G. and U. W. wrote the manuscript.

Conflict of interest

J.-P. S. is a full-time employee of Bayer Pharma AG and holds many patent applications related to sGC stimulators such as riociguat (Adempas). All other authors reported that they have no relationships to disclose relevant to the contents of this paper.

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Supporting Information

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

http://dx.doi.org/10.1111/bph.13286

Figure S1 Graphical determination of IC_{50} for Riociguat-mediated inhibition of ADP-induced human platelet aggregation. Abscissae in the main graph, Riociguat concentration [RC]; ordinatae, initial velocity of aggregation Uia. In insert, the data are replotted as the $1/U_{ia}$ vs. Riociguat concentration.