Hydrogen Sulfide–Releasing Aspirin Derivative
ACS14 Exerts Strong Antithrombotic Effects
In Vitro and In Vivo

Joachim Pircher,* Franziska Fochler,* Thomas Czermak, Hanna Mannell, Bjoern F. Kraemer, Markus Wörnle, Anna Sparatore, Piero Del Soldato, Ulrich Pohl, Florian Krötz

Objective—Hydrogen sulfide (H$_2$S)–releasing NSAIDs exert potent anti-inflammatory effects beyond classical cyclooxygenase inhibition. Here, we compared the platelet inhibitory effects of the H$_2$S-releasing aspirin derivative ACS14 with its mother compound aspirin to analyze additional effects on platelets.

Methods and Results—In platelets of mice fed with ACS14 for 6 days (50 mg/kg per day), not only arachidonic acid–induced platelet aggregation but also ADP-dependent aggregation was decreased, an effect that was not observed with an equimolar dose of aspirin (23 mg/kg per day). ACS14 led to a significantly longer arterial occlusion time after light-dye–induced endothelial injury as well as decreased thrombus formation after ferric chloride–induced injury in the carotid artery. Bleeding time was not prolonged compared with animals treated with equimolar doses of aspirin. In vitro, in human whole blood, ACS14 (25–500 µmol/L) inhibited arachidonic acid–induced platelet aggregation, but compared with aspirin additionally reduced thrombin receptor–activating peptide–, ADP–, and collagen-dependent aggregation. In washed human platelets, ACS14 (500 µmol/L) attenuated αIIbβ3 integrin activation and fibrinogen binding and increased intracellular cAMP levels and cAMP-dependent vasodilator-stimulated phosphoprotein (VASP) phosphorylation.

Conclusion—The H$_2$S-releasing aspirin derivative ACS14 exerts strong antiaggregatory effects by impairing the activation of the fibrinogen receptor by mechanisms involving increased intracellular cyclic nucleotides. These additional antithrombotic properties result in a more efficient inhibition of thrombus formation in vivo as achieved with aspirin alone. (Arterioscler Thromb Vasc Biol, 2012;32:2884-2891.)

Key Words: H$_2$S ■ aspirin ■ platelets ■ arterial thrombosis

Acetylsalicylic acid (aspirin) is one of the most frequently prescribed drugs worldwide and plays an outstanding role in the therapy of cardiovascular diseases as a platelet inhibitor. Its use is essential in the secondary prophylaxis of myocardial infarction and stroke.1 However, despite the indisputable success of this drug, its use is associated with gastrointestinal mucosal damage.2 To overcome these side effects, derivatives of NSAIDs have been developed, which release hydrogen sulfide (H$_2$S) aiming to decrease gastrointestinal complications. Indeed, in animal experiments, these new or modified drugs caused less gastric mucosal damage by affecting redox imbalance processes through increased H$_2$S/glutathione formation and increased mucosal blood flow,3,4 while exerting anti-inflammatory effects comparable with or even exceeding the effect of the respective parental drug.3,5 Several H$_2$S-related mechanisms are considered responsible for these effects.6 At lower concentrations, H$_2$S or H$_3$S donors possess anti-inflammatory properties,7–10 including inhibition of nuclear factor-kB activation,9 heme oxygenase-1 expression,11 scavenging of peroxynitrite,12 or modulation of the intracellular redox state.13,14 Because these pathways contribute to the pathophysiology of cardiovascular diseases, it is not surprising that several studies showed cardioprotective effects of H$_2$S,15,16 as well as antiatherosclerotic properties.17,18 In addition, H$_2$S plays a role in the regulation of blood pressure through vasorelaxation.19,20 Recently, the pharmacological profile of a new H$_2$S-releasing aspirin (ACS14) was described. In rodents, ACS14 exerted a similar thromboxane- and prostaglandin-suppressing activity as aspirin but showed less gastric mucosal damage.3 This effect was explained by a direct influence of H$_2$S release on cellular redox imbalance, subsequently increased glutathione formation, and by enhanced heme oxygenase-1 promoter activity resulting in lower levels of...
8-isoprostan. ACS14 also preserved endothelial function in an animal model of metabolic syndrome and ischemiareperfusion injury by modulating levels of glutathione and homocysteine.21,22

The beneficial vascular effects of ACS14 observed in animal studies raise the question whether this compound exerts the same antiaggregatory effects on platelets as aspirin or whether it may combine them with previously described antiaggregatory effects of H₂S23,24 and thereby exceed the inhibitory effects reached by aspirin alone.

We therefore investigated the ability of the H₂S-releasing aspirin derivative ACS14 to inhibit platelet activation and aggregation in vitro and ex vivo by studying different platelet activation pathways. After oral treatment of mice with ACS14, we further assessed the effects of ACS14 on thrombus formation in vivo through intravital microscopy in arterioles of the skin as well as in the carotid artery.

**Materials and Methods**

Detailed descriptions of all experiments as well as lists of materials, chemicals, and antibodies used in this study are provided in the online-only Data Supplement.

**Blood Donors and Animals**

All blood donors were healthy volunteers, who had given written consent and had not taken any medications for at least 10 days. The investigation conforms to the principles outlined in the Declaration of Helsinki. All animal experiments were conducted in wild-type C57BL/6 mice. Surgical procedures were performed under short-term anesthesia using midazolam, fentanyl, and medetomidine hydrochloride in accordance with the German animal protection law and conform to the Directive 2010/63/EU of the European Parliament. For ex vivo experiments, blood was obtained by cardiac puncture or drawn from the inferior vena cava in anesthetized wild-type C57BL/6 mice.

**Drug Administration**

ACS14 or aspirin was dissolved in dimethyl sulfoxide and further diluted in aqueous buffers. In all control experiments, the same antiaggregatory effects on platelets as aspirin or collagen (3.2 µg/mL), or collagen (3.2 µg/mL). The inhibitory effect of ACS14 was compared with the aggregation decrease exerted by equimolar doses of aspirin. In contrast to ACS14, aspirin reduced only the arachidonic acid–dependent and partly the collagen-dependent aggregation. To achieve a similar decrease of the arachidonic acid–dependent aggregation, higher concentrations of ACS14 than of aspirin were required (Figure 1A). To investigate whether platelet inhibition by ACS14 could be a result of rapid H₂S release, aggregation was also measured after incubation of blood with the H₂S donor NaHS, which impaired platelet aggregation induced by several aggregatory stimuli, however to a lesser extent than ACS14 (Figure I in the online-only Data Supplement).

To assess the inhibition of cyclooxygenase (COX), TXB₂ was measured in the supernatant of washed platelets stimulated with thrombin (0.2 U/mL) or arachidonic acid (0.1 mmol/L) for 3 minutes. Whereas thrombin- as well as arachidonic acid–induced thromboxane release was completely blocked in platelets treated with aspirin (500 µmol/L for 10 minutes), treatment with ACS14 in equimolar concentration reduced this to 4±3% and 24±5% of controls, respectively (n=4; Figure 1B).

To assess the duration of platelet inhibition, blood was incubated with ACS14 or aspirin for up to 3 hours. Whereas the strong platelet inhibitory effects on thrombin receptor–activating peptide–, ADP–, and collagen-induced platelet aggregation observed after a short time of incubation declined after around half an hour, the decreased arachidonic acid–induced aggregation was not reversible within 3 hours, similar to that observed with aspirin treatment (Figure 2).

**Plasmatic Coagulation**

We also tested whether ACS14 affects the plasmatic coagulation by performing thromboelastometry. Although the clotting time (time from stimulation to onset of clot formation) was unaffected by ACS14 or aspirin, the clot formation time (time for the formation of a solid clot) was significantly longer in ACS14-treated blood on activation of the extrinsic (184±10 s versus 148±6 s with aspirin) as well as the intrinsic coagulation cascade (183±21 s versus 118±10 s; P<0.05, n=12; Figure 1C).

**Statistical Analysis**

Data were analyzed using Student two-sample or ANOVA as appropriate to compare normally distributed variables or ANOVA on ranks when normal distribution was not given (indicated in the respective figure legend). All data are expressed as mean±SEM. Differences were considered significant when the error probability was P<0.05.
Effects on Platelet Integrity
As markers of platelet viability, we assessed the mitochondrial membrane potential and the caspase activity in washed platelets treated with 500 µmol/L of ACS14 for 10 minutes. No differences regarding the mitochondrial membrane potential (as detected by JC-1 fluorescence) as well as the caspase activity were observed compared with controls or platelets treated with equimolar concentration of aspirin (Figure 3).

Platelet Aggregation and Bleeding Time After Oral Treatment With ACS14
We investigated whether oral treatment of mice resulted in similar inhibitory effects on platelet aggregation as observed in vitro in human blood. After 6 consecutive days of feeding with ACS14 (50 mg/kg per day), COX was significantly inhibited as indicated by reduced levels of the thromboxane metabolite TXB, in the urine (Figure 4B).

Platelet aggregation was measured ex vivo in whole blood from mice, which were treated with equimolar doses of ACS14 or aspirin (50 or 23 mg/kg per day, respectively). Blood was collected 2 to 4 hours after the last treatment. As expected, both treatments showed a decrease in the arachidonic acid (0.1 mmol/L)–dependent platelet aggregation. This effect seemed to be stronger in aspirin than in ACS14-treated animals; however, the difference was not significant at the sample size chosen (24±5% versus 54±15% of controls; P=0.13, n=6). ACS14 treatment additionally resulted in reduced ADP (10 µmol/L)–dependent platelet aggregation (65±5% of controls; P<0.05, n=6), which in aspirin-treated animals was similar to untreated controls (Figure 4A).

Whereas both treatment with ACS14 and aspirin prolonged the bleeding time, as assessed by the tail bleeding assay, no difference between the treatments could be detected (Figure 4C). No changes in the platelet count and white or red blood cell counts were observed after 1 week of oral treatment with aspirin, ACS14, or vehicle. In addition, no differences in serum lactate dehydrogenase levels, a marker of hemolysis or tissue damage, were found between the treatment groups (Table).

Figure 1. ACS14 had a broader effect on platelet aggregation than aspirin. A, Aggregation upon stimulation with arachidonic acid (AA, 0.5 mmol/L), thrombin receptor–activating peptide (TRAP; 32 µmol/L), ADP (6.5 µmol/L), and collagen (3.2 µg/mL) were measured in human whole blood after incubation with increasing doses of ACS14 for 5 minutes and were compared with equimolar doses of aspirin. Representative aggregation traces for 500 µmol/L of ACS14 or aspirin are shown on the right. B, Thrombin-induced (0.2 U/mL) or arachidonic acid–induced (0.1 mmol/L) thromboxane release was significantly reduced in platelets after treatment with ACS14 500 µmol/L for 5 minutes. C, Plasmatic coagulation was analyzed by thromboelastometry after incubation with 500 µmol/L ACS14 or aspirin for 5 minutes. Whereas the clotting time (CT: time from activation until onset of clotting) was unchanged, ACS14 increased the clot formation time (CFT), which reflects the time to formation of a solid clot (indicated in thromboelastographs) and is highly dependent on platelet function (*P<0.05 vs aspirin and control; n=12, ANOVA).

Thrombus Formation in Arterioles and Carotid Artery In Vivo
Next, we investigated the influence of ACS14 on thrombus formation in vivo. To this end, a light-dye–induced endothelial injury model in arterioles of the dorsal skinfold was used. The experiments were performed 2 to 4 hours after the last
treatment. Time to complete vessel occlusion upon endothelial injury was not significantly different in aspirin-treated animals (23 ± 40 s versus 258 ± 18 s in aspirin-treated mice; n=6), whereas it was prolonged to 459 ± 43 s in animals treated with ACS14 in equimolar dose (n=6, P<0.05; Figure 5A).

As a model for arterial thrombosis in larger arteries, thrombus development in the carotid artery after FeCl3 injury was analyzed. In animals orally treated with ACS14, thrombus formation was significantly slower than that in aspirin-treated animals, where initial thrombus growth was similar to controls with less thrombus stability after ≈20 minutes. Overall thrombus size within 30 minutes of injury (expressed as the area under the curve from the fluorescence intensity of the labeled thrombus) was decreased after ACS14 treatment compared with aspirin-treated animals (57 ± 12% versus 88 ± 12% of controls, respectively; P<0.05, n=5 to 6; Figure 5B; Videos I–III in the online-only Data Supplement).

Effect on Integrin Activation and Fibrinogen Binding

Effects of ACS14 on the fibrinogen receptor, αIIbβ3 integrin, whose activation is the common final pathway mediating aggregation, were assessed by flow cytometry. Thrombin- as well as ADP-dependent inside-out activation of the receptor was significantly reduced after treatment of washed platelets with ACS14 500 µmol/L for 10 minutes. In contrast, there was no such effect of aspirin. Functionally, ACS14 led to a significantly decreased binding of fluorescently labeled fibrinogen to platelets on stimulation with ADP or thrombin. In resting platelets, neither ACS14 nor aspirin altered basal activation of the fibrinogen receptor (n=6; Figure 6A and 6B).

Platelet Secretion

To investigate the effect of ACS14 on α- and dense granule secretion, surface expression of P-selectin, as well as ATP release on thrombin stimulation, was measured. Whereas no differences between ACS14 or aspirin could be detected with regard to ATP release, ACS14, but not aspirin treatment, slightly reduced P-selectin expression (n=6; Figure 6C and 6D).

VASP Phosphorylation and Intracellular cAMP

Because H2S, which is released from ACS14, has been shown to increase the intracellular concentration of cyclic nucleotides in other cells, intracellular cAMP levels were measured by ELISA in lysates of washed platelets. Compared with controls, ACS14 treatment (500 µmol/L, 10 minutes) significantly elevated cAMP levels (1950 ± 66 fmo/L/10^7 platelets versus 1479 ± 118 fmo/L/10^7 platelets in controls; P<0.05, n=5; Figure 6F). Phosphorylation of VASP as a
WBC, 103/μL platelets, the new H2S-releasing aspirin derivative ACS14.

In this study, we demonstrate that in human and murine platelets, the new H2S-releasing aspirin derivative ACS14, in contrast to aspirin, prolonged clot formation time, which represents the speed at which a solid clot is formed and is heavily dependent on platelet function. These results correspond to the in vivo observation of a markedly decreased arterial thrombus formation in arterioles and in large arteries. Furthermore, we show that attenuated activation of the αβIibβ3 integrin by ACS14 and increase of intracellular cyclic nucleotides contribute to the antithrombotic effects.

Our in vitro findings in human whole blood demonstrate that ACS14, like aspirin, is a potent inhibitor of platelet aggregation. The antithrombotic and antiaggregatory effect of low-dose aspirin is attributed to the permanent inhibition of COX-1–dependent TXA2 formation in platelets. ACS14 also effectively inhibits platelet thromboxane release, but compared with aspirin higher doses are required to achieve similar COX inhibition. This could be because of a less effective release of the aspirin molecule from the ACS14 compound or because of a more rapid deacetylation, which has been observed in pharmacokinetic studies. In contrast to aspirin, a decrease in platelet aggregation by ACS14 is not limited to COX inhibition. ACS14 reduced thrombin-, ADP-, and collagen-dependent platelet aggregation, which are not relevant targets of unconjugated aspirin. These additional effects can, in part, be explained by a rapid release of H2S, because platelet inhibition by the H2S donor NaHS, where H2S release occurs rapidly, was qualitatively similar to the inhibition exerted by ACS14, but quantitatively less pronounced. This in turn may be owing to differences in the kinetics of the H2S release but could also arise from the remaining thiol groups of ACS14.

Our in vitro findings in human whole blood demonstrate that ACS14 reduced platelet aggregation in mice after oral treatment. C57/BL6 mice were fed with ACS14 50 mg/kg per day or equimolar dose of aspirin (ie, 23 mg/kg per day) for 6 consecutive days. Whereas aspirin decreased only arachidonic acid (AA, 0.1 mmol/L)-induced platelet aggregation, ACS14 additionally reduced aggregation upon stimulation with ADP (6.5 µmol/L). Thromboxane (TX) B2 in the urine was significantly reduced by ACS14, indicating effective cyclooxygenase (COX) inhibition. This could be because of a less effective release of the aspirin molecule from the ACS14 compound or because of a more rapid deacetylation, which has been observed in pharmacokinetic studies. In contrast to aspirin, a decrease in platelet aggregation by ACS14 is not limited to COX inhibition. ACS14 reduced thrombin- and TXA2- and collagen-dependent platelet aggregation, which are not relevant targets of unconjugated aspirin. These additional effects can, in part, be explained by a rapid release of H2S, because platelet inhibition by the H2S donor NaHS, where H2S release occurs rapidly, was qualitatively similar to the inhibition exerted by ACS14, but quantitatively less pronounced. This in turn may be owing to differences in the kinetics of the H2S release but could also arise from the remaining thiol groups of ACS14.

The striking inhibitory effects on several platelet-activating pathways exerted by ACS14 raise questions about unspecific and eventually deleterious effects of the compound on platelets. However, even higher doses of ACS14 neither affected platelet mitochondrial membrane potential, an indicator of cell viability, nor activation of cell death–executing caspases. Also, hematologic parameters in animals treated with ACS14 such as platelet and blood cell counts or lactate dehydrogenase levels remained unchanged by treatment with the compound.

In full agreement with our in vitro findings in human whole blood, oral treatment with ACS14 in mice, which has previously been shown to reduce aspirin-dependent gastrointestinal damage in rodents, potently inhibited COX and consequently arachidonic acid–dependent platelet aggregation. Platelet aggregation in blood drawn from ACS14- but not aspirin-treated animals was also significantly decreased on ADP stimulation, indicating that the COX-independent platelet

**Table. Hematologic Parameters and Cytotoxicity as Assessed by LDH Levels Were Not Changed by ACS14**

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>Aspirin</th>
<th>ACS14</th>
</tr>
</thead>
<tbody>
<tr>
<td>WBC, 10^9/μL</td>
<td>2.9±0.8</td>
<td>2.4±0.4</td>
<td>2.8±0.6</td>
</tr>
<tr>
<td>Hct, %</td>
<td>45±4</td>
<td>47±4</td>
<td>45±3</td>
</tr>
<tr>
<td>Plt, 10^3/μL</td>
<td>1266±64</td>
<td>1275±158</td>
<td>1311±254</td>
</tr>
<tr>
<td>LDH, U/L</td>
<td>19±7</td>
<td>20±6</td>
<td>17±4</td>
</tr>
</tbody>
</table>

Mice were treated with equimolar doses of aspirin (23 mg/kg per day), ACS14 (50 mg/kg per day), or vehicle for 6 consecutive days (n=4–6 animals per group). WBC indicates white blood cells; Hct, hematocrit; Plt, platelets; LDH, lactate dehydrogenase.

but, unlike aspirin, also decreases ADP-, collagen-, and thrombin-induced aggregation. These COX-independent effects were observed both after short incubation of whole blood in vitro and after continuous oral ingestion in mice. Consequently, ACS14, in contrast to aspirin, prolonged clot formation time, which represents the speed at which a solid clot is formed and is heavily dependent on platelet function. These results correspond to the in vivo observation of a markedly decreased arterial thrombus formation in arterioles and in large arteries. Furthermore, we show that attenuated activation of the αβIibβ3 integrin by ACS14 and increase of intracellular cyclic nucleotides contribute to the antithrombotic effects.

**Discussion**

In this study, we demonstrate that in human and murine platelets, the new H2S-releasing aspirin derivative ACS14 not only inhibits arachidonic acid–dependent aggregation but, unlike aspirin, also decreases ADP-, collagen-, and thrombin-induced aggregation. These COX-independent effects were observed both after short incubation of whole blood in vitro and after continuous oral ingestion in mice. Consequently, ACS14, in contrast to aspirin, prolonged clot formation time, which represents the speed at which a solid clot is formed and is heavily dependent on platelet function. These results correspond to the in vivo observation of a markedly decreased arterial thrombus formation in arterioles and in large arteries. Furthermore, we show that attenuated activation of the αβIibβ3 integrin by ACS14 and increase of intracellular cyclic nucleotides contribute to the antithrombotic effects.

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inhibitory effects of ACS14 were preserved even after oral treatment and that findings in mouse and human platelets were alike.

In an in vivo thrombosis model, in the microcirculation of the dorsal skin, in which the endothelium is specifically damaged by photoactivation of a fluorochrome, ACS14, but not aspirin, significantly prolonged the time to complete vessel occlusion by a thrombus. This advantageous effect of ACS14 also reflects in vivo the broader antiaggregatory and antithrombotic effects of ACS14 compared with aspirin.

Collagen, the most prominent platelet activator in the subendothelium, and thrombin, generated by tissue factor exposure, likely represent important initial platelet activators at the site of endothelial injury on atherosclerotic plaque rupture, whereby the autoactivating substances, ADP and TXA$_2$, also play key roles. As atherothrombotic events predominantly occur in larger vessels, we proceeded to investigate thrombus formation in the carotid artery. Indeed, also in this model, ACS14 significantly reduced thrombus size after vessel injury, whereas aspirin in an equimolar dose primarily reduced the stability of the thrombus.

How can the broad antiaggregatory and antithrombotic effects of ACS14 in functional in vitro assays and in the in vivo models be explained? The fact that inhibition of platelet function with ACS14 is observed with several platelet-activating stimuli points toward common mechanisms that are being central for regulatory platelet aggregation processes. Among these, the integrin $\alpha$IIb$\beta$3, which functions as the fibrinogen receptor, plays an outstanding role. Indeed, we found that ACS14 significantly diminished the activation of the fibrinogen receptor, which could explain the common strong decrease of platelet aggregation on the stimulation of several activation pathways.

H$_2$S donors have recently been shown to inhibit phosphodiesterase and activate adenylate cyclase in vascular smooth muscle cells. cAMP activates the cAMP-dependent protein kinase, protein kinase A, which inhibits platelets by phosphorylation of VASP and other target proteins. VASP regulates actin dynamics, and the increase of the phosphorylated form is associated with decreased platelet aggregation and decreased activation of $\alpha$IIb$\beta$3 integrin. The observed increase of phosphorylation of VASP at Ser157 as well as intracellular cAMP levels in platelet lysates after treatment with ACS14 constitutes a possible pathway responsible for the inhibitory effects of ACS14. However, additional modification of other extracellular thiol-containing receptors, such as the ADP receptor P2Y$_{12}$ or the collagen receptor GPVI, by ACS14 cannot be excluded.

Our findings may be of considerable therapeutic interest. Presently, antithrombotic therapy in cardiovascular diseases is mainly based on the inhibition of TXA$_2$, formation by aspirin and the blockade of the P2Y$_{12}$ receptor. However, this dual therapy is associated with a higher risk of bleeding events, by ACS14, which offers a new mechanistic profile, therefore deserves further clinical investigation. This holds especially true for patients with coronary artery disease, because these...
show greater ADP-induced activation of the fibrinogen receptor, and higher doses of aspirin are required to affect ADP- and collagen-induced aggregation.46

In conclusion, our findings indicate that ACS14 strongly inhibits platelet aggregation by affecting fibrinogen receptor activation and increasing intracellular cAMP levels, and thereby attenuating arterial thrombus formation in vivo. Altogether, ACS14, in addition to spare the gastric mucosa,1 seems to be a drug with broad-spectrum effects on platelet function that could prove beneficial in antiaggregatory therapy in the future.

**Figure 6.** ACS14 decreased αIIbβ3 integrin activation and fibrinogen binding. A, In washed human platelets, ACS14 (500 µmol/L for 10 minutes) led to reduced activation of the αIIbβ3 integrin on stimulation with thrombin (0.2 U/mL) or ADP (5 µmol/L) as detected by PAC-1 antibody leading to decreased fibrinogen binding (B). C, Whereas surface expression of P-selectin on stimulation with thrombin (0.2 U/mL) was slightly decreased by ACS14, ATP release was not changed (D). E, ACS14 increased phosphorylation of VASP and (F) intracellular levels of cAMP. Histograms or blots are representative for 5 to 6 independent experiments (*P<0.05 vs aspirin and control, ANOVA; t test for E).

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This article contains part of the doctoral thesis of Franziska Fochler to be submitted to the medical faculty of the Ludwig-Maximilians-University.

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Disclosures
None.

References
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LEGENDS FOR VIDEO FILES

VIDEO 1
Thrombus formation in the carotid artery upon ferric chloride injury in control-treated animal.
Thrombus formation was visualized using DIOC₆ and observed for 30 minutes. In time lapse video one minute corresponds to the real time of 30 minutes.

VIDEO 2
Thrombus formation in the carotid artery upon ferric chloride injury in aspirin- (23 mg/kg/d for 6 days) treated animal.
Thrombus formation was visualized using DIOC₆ and observed for 30 minutes. In time lapse video one minute corresponds to the real time of 30 minutes.

VIDEO 3
Thrombus formation in the carotid artery upon ferric chloride injury in ACS14- (50 mg/kg/d for 6 days, equimolar to aspirin 23 mg/kg/d) treated animal.
Thrombus formation was visualized using DIOC₆ and observed for 30 minutes. In time lapse video one minute corresponds to the real time of 30 minutes.
SUPPLEMENTAL MATERIAL

The hydrogen sulfide releasing aspirin-derivative ACS14 exerts strong antithrombotic effects in vitro and in vivo

Joachim Pircher, MD 1, 2, Franziska Fochler, MS 2, Thomas Czermak, MS 2, Hanna Mannell, PhD 2, Bjoern Kraemer, MD 1, Markus Wörmle, MD 1, Anna Sparatore, PhD 3, Piero Del Soldato, PhD 3, Ulrich Pohl, MD 2, Florian Krötz, MD 1, 4

1 Medizinische Klinik und Poliklinik IV, Klinikum der LMU, München; 2 Walter-Brendel-Centre of Experimental Medicine, University of Munich, Germany and Munich Heart Alliance; 3 Dipartimento di Scienze Farmaceutiche, Università degli Studi di Milano, Italy; 4 Invasive Cardiology, Klinikum Starnberg, Starnberg, Germany
DETAILED METHODS SUPPLEMENT

Chemicals and antibodies

ACS14 was prepared as previously described, reagents for Multiplate analysis were from Dynabyte (Munich, Germany), and reagents for thrombelastometry (ROTEM) were from Pentapharm AG (Basel, Switzerland). Prostacyclin analog (iloprost) was from Schering (Berlin, Germany). The anaesthetics Midazolam, Fentanyl and Medetomidinhydrochloride were from Ratiopharm, CuraMED Pharma and Pfizer (all Germany) respectively. APC-labeled anti-P-Selectin Antibody, FITC-labeled PAC-1 antibody and respective isotype controls were from BD (Franklin Lake, USA), anti-VASP-antibody was from Enzo Life Sciences (Lörrach, Germany), anti-phospho-VASP (Ser157) was from Cell Signaling Technology (Danvers, MA, USA). APC-labeled fibrinogen was from Invitrogen (Carlsbad, CA, USA). All other substances were obtained from Sigma Aldrich (Taufkirchen, Germany).

Platelet preparation

Platelet rich plasma was obtained by centrifugation of anticoagulated (3.13% sodium citrate) whole blood at 340 \(g\) for 15 minutes. After another centrifugation step at 600 \(g\) for 10 minutes in the presence of 2 ng/mL Ilomedin, platelets were washed and resuspended in calcium-free modified Tyrode buffer (138 mmol/L NaCl, 2.7 mmol/L KCl, 12 mmol/L NaHCO\(_3\), 400 mmol/L Na\(_2\)HPO\(_4\), 1 mmol/L MgCl\(_2\), 5 mmol/L D-glucose, and 5 mmol/L HEPES) and adjusted to the concentration required for the respective experiment. Platelet counts were obtained using a resistance particle counter (Coulter Z2, Beckman Coulter, Krefeld, Germany).

Collection of murine blood

For mice studies blood was collected from the heart or the inferior vena cava in anesthetized mice with a syringe containing the anticoagulant required for the respective experiment.

Platelet aggregation studies

Platelet aggregation in whole blood was performed by impedance aggregometry with the Multiplate (multiple platelet function analyzer) assay (Dynabyte, Munich, Germany) according to the manufacturer’s protocol. Blood samples were drawn into tubes containing hirudin (13 \(\mu\)g/mL) to avoid alterations in physiological calcium concentration. The samples were allowed to rest for 30 minutes
and aggregation studies were performed 30 to 180 minutes after blood had been drawn. An aliquot of 300 µL of whole blood was added to each test cell, diluted with 300 µL saline solution (0.9%) and incubated with ACS14 or aspirin at 37°C for 5 minutes under constant stirring (800/min) with a poly-tetra-fluoroethylene coated magnetic stirrer at 37°C. Aggregation was started by adding 20 µL of the appropriate agonist solution provided by the manufacturer under constant stirring (800/min) at 37°C. Changes in impedance expressed as aggregation amplitudes were recorded over 6 minutes in duplicates and results were expressed as mean arbitrary aggregation units (AU).

Assessment of plasmatic coagulation

Parameters of the plasmatic coagulation were assessed by thrombelastometry (ROTEM). According to the instructions of the manufacturer 20 µL of 200 mmol/L CaCl₂ for re-calcification and 20 µL of the respective activation reagent (human recombinant tissue factor for the extrinsic system (EXTEM) and partial thromboplastin phospholipid made of rabbit brain for the intrinsic system (INTEM)) were added into pre-warmed reaction cups of the device. Then, 300 µL of citrated (3.13% sodium citrate) human whole blood was added and, after a semi-automated mixing step, the cup holder was placed in the measuring position of the device. The measurement was recorded for at least 20 minutes and clotting time (CT; time to onset of clot formation) and clot formation time (CFT; time from onset of clot formation to a clot firmness of 20 mm) were analyzed for the extrinsic as well as for the intrinsic activation.

Platelet thromboxane release

Washed platelets in a concentration of 100,000/µL were stimulated with arachidonic acid (0.1 mmol/L) or thrombin (0.2 U/mL) for 3 minutes and then centrifugated for one minute at 3,000 g in the presence of 10 µg/mL indomethacin. Concentrations of TXB₂ were measured in the supernatant by ELISA (Enzo Life Sciences, Lörrach, Germany).

Markers of platelet viability

As an indicator of platelet viability the mitochondrial membrane potential was assessed by JC-1 fluorescence and caspase activity was measured by a poly caspase assay kit (Immunochemistry, Bloomington, USA) according to the protocol of the manufacturer. Washed platelets were incubated
with JC-1 or fluorescent inhibitors of activated caspases analyzed by a FACS Canto II flow cytometer (BD, Franklin Lake, USA).

**Urine TXB₂**

Mouse urine samples were obtained by puncture of the bladder of euthanized mice. Indomethacin 10 µg/mL was immediately added and samples were stored at -80°C until further analysis. TXB₂ and creatinine as a reference parameter for urine concentration were measured by ELISA or enzymatic creatinine detection kit (both Enzo Life Sciences, Lörrach, Germany) respectively according to the manufacturers protocol.

**Blood parameters in mice**

Blood cell counts in murine whole blood were measured using a Beckmann Coulter Counter (Coulter Z2, Beckman Coulter, Krefeld, Germany). LDH levels as a parameter of hemolysis or tissue damage were determined in the serum of mice by a commercially available LDH detection kit (Cayman, Ann Arbor, USA). Serum was obtained by centrifugation of clotted whole blood at 3000 g for 10 minutes and stored at -80°C until analysis.

**Bleeding time**

To assess bleeding time a 5 mm segment of the tail of anesthetized mice was removed with a razor blade. The tail was immediately immersed in 0.9% isotonic saline at 37°C, and the time required to stop spontaneous bleeding was measured.

**Intravital assessment of arteriolar thrombus formation**

Arteriolar thrombus formation in vivo was assessed in the dorsal skinfold chamber microcirculatory model in mice using a light dye injury model.

The dorsal skinfold chamber was prepared in mice as described previously ². Briefly in anaesthetized mice the hair on the back was removed by a hair clipper (Aesculap) and chemical depilation (Pilcamed, Schwarzkopf). After disinfection with alcohol 70% subcutaneous vessels were visualized diaphanoscopically and an extra manufactured chamber made up of two inversed plates of titanium was implanted under sterile condition. In this chamber a circular window is building the frame for an extra cut circular coverslip (ø 11,7 mm, Menzel, Braunschweig), which covers a circular area where the skin was removed on one side in a way that the skin of the opposite side is attached via adhesion
and its blood vessels can be observed macroscopically and microscopically. All experiments were
performed at a minimum of 24 hours after chamber implantation. While animals showing abnormal
flow were excluded from the experiments, the animals fulfilling the criteria for an intact
microcirculation (accurate arteriolar blood flow, absence of surgery-related artefacts) underwent
carotid artery catheterization for application of the dyes. The surgical procedure of catheterization was
performed under the same short time anaesthesia protocol as described above for the implantation of
the dorsal skinfold chamber. From the point of taking off the coverslip of the skinfold chamber and
during the length of the experiment the tissue was kept warm and moist by continuous superfusion
with pre-warmed (37°C) Krebs-Henseleit-solution pH 7.4 (118 mmol/L NaCl, 4.8 mmol/L KCl, 2.5
mmol/L CaCl₂, 1.2 mmol/L MgSO₄, 25 mmol/L NaHCO₃, 1.2 mmol/L KH₂PO₄). Intravital
fluorescence microscopy was performed using a Zeiss Axiotech Vario microscope (Zeiss, Göttingen,
Germany).

After injection of 4 µL/g of 5% FITC-dextran (150,000 MW) via a carotid catheter photoactivation
was initiated by exposing the vessel to a 100 W mercury lamp (Fluoarc HBO100, Carl Zeiss
Germany) using the respective fluorescence filter. Intravital fluorescence microscopy was performed
using a Zeiss Axiotech Vario microscope (Zeiss, Göttingen, Germany) and thrombus formation was
recorded with a digital camera (AxioCam HSm, Carl Zeiss Germany) in 4-6 arterioles per mouse with
at least 40 µm in diameter. For quantification the time to complete thrombotic vessel occlusion
resulting in flow cessation was determined.

**Intravital thrombus formation in the carotid artery (FeCl₃-injury)**

Thrombus formation *in vivo* was assessed in the carotid artery in a model previously described³ with
minor modifications. In anesthetized mice (same short time anaesthesia protocol as described above
for the implantation of the dorsal skinfold chamber) a polyethylene catheter was placed in the left
jugular vein. The fluorescent dye DiOC₆ in 100 µmol/L solution (5 µL/g of body weight) was injected
into the jugular vein to allow visualization of the thrombus. After an equilibration time of 10 minutes a
segment of the right carotid artery was exposed and injury was induced by the topical application of
FeCl₃ for 2 minutes (Whatman paper 1 mm² soaked with 2 µL of 10% FeCl₃). The artery was then
rinsed with saline, and thrombus formation was monitored for 30 minutes by placing the carotid artery
under a fluorescence microscope equipped with a camera (AxioScope; Carl Zeiss). Fluorescent images were acquired sequentially (1 image/second) and quantified using AxioVision 4.7 imaging software (Carl Zeiss). Thrombus size was calculated from the fluorescence signal of a single image and cumulative thrombus size over 30 minutes was expressed as area under the curve (AUC).

**Flow cytometry**

P-Selectin, activated αIIbβ3-integrin and fibrinogen-binding were measured on washed human platelets by flow cytometry using a FACS Canto II flow cytometer (BD, USA). Resting and thrombin or ADP (0.2 U/mL or 5 µmol/L respectively for 3 minutes) stimulated platelets were stained with APC-labeled P-Selectin Antibody, FITC-labeled PAC-1 antibody or respective isotype controls for 30 minutes at 37°C, fixed with 1% formaldehyde before the fluorescence was analyzed.

For investigation of fibrinogen binding platelets were pre-incubated with APC-labeled fibrinogen (150 µg/mL) before stimulation and fixation.

**Platelet ATP release**

Platelet ATP release was measured by Chemiluminescence. Therefore washed platelets in a concentration of 250,000/µL were incubated with Luciferin-Luciferase reagent (0.2 µmol/L, 2000 U/mL respectively; Chrono-Lume, Probe & go Labordiagnostica GmbH; Osburg, Germany) for 5 minutes and then stimulated with thrombin. Photon emission over 10 minutes was recorded by a LB 9507 Luminometer (Berthold, Germany) and ATP concentration was calculated using ATP standard solution.

**VASP-phosphorylation**

Phosphorylation of VASP was assessed by Western blotting in human whole platelet lysates. Lysis and Western blot analysis were performed as described previously. Membranes were blocked by incubation with either 5% (w/v) BSA (phospho-VASP-antibody) in TBSt (Tris-buffered saline with Tween) or 2.5% (w/v) non-fat dried milk powder in TBSt. Primary antibodies were diluted in either 5% (w/v) BSA or 2.5% (w/v) non-fat dried milk powder in TBSt, respectively. Densiometric analysis of the blots was performed digitally using WASABI Software. Five minutes of stimulation with Iloprost at 5 nmol/L was used as positive control and reference for each donor.
**cAMP measurement**

Intracellular cAMP levels were assayed with a commercially available enzyme immunoassay kit (GE Healthcare) in lysates of human washed platelets. $4 \times 10^8$ platelets were then lysed using the lysis reagent provided by the immunoassay kit and cAMP was measured according the manufacturers protocol. All measurements were carried out in duplicates and with different platelet donors. The results were expressed in fmol/10$^7$ platelets.

**SUPPLEMENTAL REFERENCES**

$\text{H}_2\text{S}$ inhibited platelet aggregation to a lesser extent than ACS14. (A) Human whole blood was incubated with increasing doses of the $\text{H}_2\text{S}$-donor NaHS for 5 minutes and platelet aggregation was measured by impedance aggregometry upon stimulation with ADP (6.5 µmol/L), TRAP (32 µmol/L), arachidonic acid (AA, 0.5 mmol/L) and collagen (3.2 µg/mL). (B) Platelet inhibition was observed in several pathways of activation but was significantly less as compared to equimolar concentrations of ACS14 (n=5) indicating additional mechanisms than the release of $\text{H}_2\text{S}$ contribute to the platelet inhibitory effects.