An Activation-Specific Platelet Inhibitor That Can Be Turned On/Off by Medically Used Hypothermia

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Objective—Therapeutic hypothermia is successfully used, for example, in cardiac surgery to protect organs from ischemia. Cardiosurgical procedures, especially in combination with extracorporeal circulation, and hypothermia itself are potentially prothrombotic. Despite the obvious need, the long half-life of antithrombotic drugs and thus the risk of postoperative bleedings have restricted their use in cardiac surgery. We describe here the design and testing of a unique recombinant hypothermia-controlled antiplatelet fusion protein with the aim of providing increased safety of hypothermia, as well as cardiac surgery.

Methods and Results—An elastin-mimetic polypeptide was fused to an activation-specific glycoprotein (GP) IIb/IIIa-blocking single-chain antibody. In silico modeling illustrated the sterical hindrance of a β-spiral conformation of elastin-mimetic polypeptide preventing the single-chain antibody from inhibiting GPIIb/IIIa at 37°C. Circular dichroism spectra demonstrated reverse temperature transition, and flow cytometry showed binding to and blocking of GPIIb/IIIa at hypothermic body temperature (≤32°C) but not at normal body temperature. In vivo thrombosis in mice was selectively inhibited at hypothermia but not at 37°C.

Conclusion—This is the first description of a broadly applicable pharmacological strategy by which the activity of a potential drug can be controlled by temperature. In particular, this drug steerability may provide substantial benefits for antiplatelet therapy. (Arterioscler Thromb Vasc Biol. 2011;31:00-00.)

Key Words: antibodies | antiplatelet drugs | platelet receptor blockers | thrombosis | hypothermia

Antithrombotic treatment is one of the most often used medical therapies, with major benefits for patients. However, the side effects associated with antithrombotic therapy can cause severe and even life-threatening complications. Antiplatelet drugs are particularly troublesome because of their long half-life and the lack of effective antidotes. In cardiac surgery, especially when combined with extracorporeal circulation, platelet inhibition could be of major benefit. However, because postoperative bleeding complications with long-acting antiplatelet drugs are feared, therapeutic platelet inhibition is preferentially avoided. We describe here a novel pharmacological strategy that uses hypothermia to tightly control and thus allow broadening of the application of platelet inhibition and potentially reducing bleeding complications.

Therapeutic hypothermia is already widely used to reduce oxygen requirements and to protect organs from ischemia. In cardiac surgery, hypothermia is typically established temporarily with the heart-lung machine during coronary artery bypass grafting (CABG), whereas deep hypothermic circulatory arrest is an integral component of surgery for congenital heart disease and is used in adult patients for the repair of thoracic aortic dissections, as well as in neurological operations for the treatment of cerebral aneurysm. However, therapeutic hypothermia used in clinical settings, such as cardiac surgery, has also been associated with platelet activation, aggregation, sequestration, and (micro)vascular thrombus formation. This may result in fatal thrombotic events and severe bleeding complications, in particular postoperatively. Therefore, these complications indicate a need for a pharmacological approach that prevents hypothermia-associated platelet activation and after rewarming provides postoperatively re-installed full platelet function.

Inhibiting the glycoprotein (GP) IIb/IIIa (αIIbβ3, CD41/CD61) represents one of the most effective strategies for platelet inhibition. In the setting of cardiac surgery, preop-
erative treatment with the commercially available GPIIb/IIIa blockers tirofiban and eptifibatide, which block GPIIb/IIIa irrespective of its conformation, has been reported to preserve platelet function during cardiac pulmonary bypass. However, tirofiban and eptifibatide are not optimal candidates for the desired indication because of their half-lives of 1.5 to 2 hours. Furthermore, a significant side effect of conformation-unspecific GPIIb/IIIa blockers is their inhibitory effect on all circulating platelets, which also carries a risk for bleeding complications. We have previously described a single-chain antibody (scFv) directed against GPIIb/IIIa that selectively blocks the activated form of the receptor only, potentially providing additional advantages compared with the already successfully clinically used GPIIb/IIIa blockers. In addition, this recombinant antibody can be genetically manipulated, thereby providing an ideal basis for new pharmacological strategies. Therefore, we hypothesized that the fusion of this anti-GPIIb/IIIa with a temperature-sensitive molecule might provide temperature-dependent control of platelet inhibition. The molecule chosen for this approach is an elastin-mimetic polypeptide (EMP), which belongs to a family of repetitive polypeptides derived from consensus sequences, including tetrapeptide (VPGG), pentapeptide (VPGVG) or (VPAVG), and hexapeptide (APGVGV) repeat motifs of the primary structure of mammalian tropoelastin. These peptides are characterized by an inverse temperature transition, where the largely unstructured elastin repeats gain β-sheet structure at temperatures greater than the transition temperature (Tm). This specific property has prompted the promising development of several EMP-based biotechnological applications, such as protein purification and nanopartnering, temperature-dependent linker peptides, hyperthermia-mediated targeting of tumors, and drug delivery.

In this study, we describe the successful fusion of EMP to an activation-specific GPIIb/IIIa-blocking scFv. In vitro and in vivo experiments show that the EMP-scFv enables selective hypothermia-induced antiplatelet therapy. We propose a structural model of the EMP-scFv fusion protein to illustrate how the β-spiral conformational transition of EMP prevents scFv from inhibiting GPIIb/IIIa at 37°C but not at hypothermia. Overall, this is the first report on the design and testing of a highly potent antiplatelet drug that can be turned on/off by medically used hypothermia.

Methods
For detailed methods, please see the supplemental materials (available online at http://atvb.ahajournals.org).

Generation of the EMP-scFv Fusion Construct
A recombinant clone encoding EMP and a short linker sequence was cloned into the pET39b(+)-spiral conformational transition of EMP prevents scFv from inhibiting GPIIb/IIIa at 37°C but not at hypothermia. Overall, this is the first report on the design and testing of a highly potent antiplatelet drug that can be turned on/off by medically used hypothermia.

Methods
For detailed methods, please see the supplemental materials (available online at http://atvb.ahajournals.org).

Generation of the EMP-scFv Fusion Construct
A recombinant clone encoding EMP and a short linker sequence was cloned into the pET39b(+)-vector and then subcloned into pHOG-21 bacterial expression vector already containing scFv cDNA.

Expression of EMP-scFv Construct in Escherichia coli and Protein Purification
The EMP-scFv fusion protein was expressed in E. coli, and the insoluble fraction was purified using Ni2+-agarose beads.

Circular Dichroism and Thermal Transition Profile Monitoring
Circular dichroism (CD) spectra were recorded on a Jasco J-810 spectropolarimeter, and thermal transition curves were plotted using the mean residue ellipticity.

In Silico Structural Analysis of EMP-scFv Fusion Peptide
A model of scFv was generated using SWISS-MODEL server and Protein Data Bank entry 3b9D. The structure was analyzed using PROCHECK and a Tripos force field. A β-spiral model of EMP was constructed with the Biopolymer module in Sybyl8.1.

Flow Cytometry
Binding to and blocking of GPIIb/IIIa was evaluated using flow cytometry as previously described.

Platelet Aggregometry and Targeting of EMP-scFv to Platelet Aggregates Under Physiological Flow Conditions at Different Temperatures
The effects of EMP-scFv on platelet aggregation were analyzed using a BioRad Benchmark plate reader according to a previously established method, and the microscopic analysis of platelet aggregates was assessed using an Olympus CKX41 microscope. Images of flow chamber experiments were taken with an Olympus IX51 microscope with an XM10 camera.

In Vivo Functional Evaluation of Antithrombotic Efficacy of EMP-scFv at Different Temperatures
Thrombus development was induced in carotid arteries and jugular veins of mice by local vessel injury using ferric chloride, and the antithrombotic efficacy of EMP-scFv was analyzed following a previously established protocol.

Statistical Analysis
Data are presented as mean±SD for the indicated numbers of mice and blood donors.

Results
Construction, Expression, and Purification of EMP-scFv
Cloning an EMP cDNA upstream of the scFv in pHOG-21 bacterial expression vector resulted in a 2115-bp fusion construct (EMP-scFv) (Figure 1A), encoding a protein consisting of 704 amino acids, with a molecular weight of approximately 70 kDa (Figure 1B). Western blotting using a horseradish peroxidase–coupled anti-His(6)-tag monoclonal antibody resulted in the detection of a single band, further...
confirming that the protein visualized by SDS-PAGE was EMP-scFv (Figure 1C).

CD Spectra Profiles and Thermal Transition Profiles of Recombinant Constructs

The structural feature of the scFv is a β-sheet. As anticipated, no significant conformational changes were observed in CD as the temperature increased up to 35°C (Figure 2A). The EMP displayed temperature-dependent conformational rearrangement from the random coil conformation (negative ellipticity near 198 nm) to the β-turn signature (negative ellipticity near 220 nm) (Figure 2B). Likewise, significant conformational changes could be detected in the CD spectra of EMP-scFv fusion molecule in that the random coil (negative ellipticity near 200 nm) was rapidly replaced with the putative β-turn signature (negative ellipticity near 220 nm) as the temperature approached the transition point (Figure 2C). The negative minimum ellipticity of the fusion protein at high temperature was slightly shifted from that of EMP, indicating an interaction between 2 components of the fusion protein.

The transition curves of the EMP demonstrated a typical unfolding/folding model representing a 2-state transition (Figure 2D). The mathematical fitting of the CD data allowed the determination of the $T_t$ resulting from conformational change of EMP as the temperature is increased. The mathematical fitting of the CD data to the van’t Hoff equation allowed the determination of the $T_t$ resulting from conformational change of EMP and EMP-scFv as the temperature was increased. As Reiersen et al. reported, the transition temperature was calculated using a linear van’t Hoff plot of $\ln K$ (equilibrium constant, $K$, where $K = [\theta]_{\text{obs}}^{\text{eq}}/[\theta]_{\text{obs}}^{\text{fr}}$ as a function of $1/T$ and from the relation $T = \Delta H/\Delta S$.

The $T_t$ calculation indicated the consistent $T_t$ of approximately 26°C for 198 and 220 nm. In contrast, different $T_t$ values of EMP-scFv for the disappearance of the random coil and the appearance of the β-turn structure were clearly obtained from fitting of the CD data at 200 nm (15°C) and at 215 nm (28°C), strongly suggesting the presence of a multistate transition, including conformational change of EMP domain and hydrophobic interaction between the hydrophobic residues on the surface of antibody and the EMP within the fusion protein (Figure 2E). This thermal transition curve of EMP-scFv also suggests that structural change and hydrophobic collapse in the fusion were completed at temperatures higher than 35°C.

Proposed Structural Models of EMP-scFv Fusion Protein

Figure 3 shows the proposed structural model from one of the top docks for the EMP-scFv fusion construct after the conformational transition has occurred at 37°C. The location
of the β-spiral EMP obstructs the RXD motif in the CDR3 region of the heavy chain, which in turn inhibits binding of the scFv to GPIIb/IIIa (Figure 3A). This is further confirmed by a 90° rotation around the y-axis, which demonstrates the complementarity of the β-spiral of EMP for the antibody (Figure 3B), particularly when displayed on a larger scale (Figure 3C).

**Temperature-Specific Binding of EMP-scFv and Inhibition of Fibrinogen Binding to Platelets**

The function of the scFv component of the fusion molecule was evaluated by flow cytometry. A significantly higher level of EMP-scFv binding to human activated platelets was evident when experiments were conducted at 22°C, whereas the binding of the fusion protein to activated platelets was totally abolished when the temperature of the assay was increased to 37°C (Figure 4A). No binding of the EMP-scFv recombinant protein to resting platelets was detected, regardless of the temperature used in the experiment (Figure 4A).

In a concentration-dependent manner, EMP-scFv significantly reduced the level of fibrinogen binding to human activated platelets at 22°C, reaching almost the same inhibitory levels as observed with the positive control abciximab (ReoPro), which is a clinically used humanized Fab fragment.
blocking GPIIb/IIIa (Figure 4B). Binding of fibrinogen to activated platelets by EMP-scFv was not inhibited at 37°C (Figure 4B). Fibrinogen did not bind to resting platelets regardless of the temperature used (Figure 4B). To further demonstrate the temperature-dependent blockade of the ligand-binding pocket of GPIIb/IIIa by EMP-scFv, the binding of PAC-1, which is a monoclonal antibody that binds selectively to the ligand binding pocket of activated GPIIb/IIIa, was evaluated. EMP-scFv inhibited the binding of PAC-1 to activated platelets at 22°C but not at 37°C (Supplemental Figure I). Rewarming experiments demonstrated that after binding to activated platelets at 22°C, EMP-scFv did not dissociate from GPIIb/IIIa on increasing the temperature to 37°C (Figure 4C). Furthermore, flow cytometry confirmed the ability of EMP-scFv to bind fibrinogen (Supplemental Figure II) and to inhibit the binding of fibrinogen (Supplemental Figure IIB) to human activated platelets at 32°C, a mild form of hypothermia increasingly used to treat out-of-hospital cardiac arrests.46

To confirm that the blocking effect observed was due to the binding of the scFv component of the fusion protein to the activated platelets, we generated EMP alone following the same approach used to purify EMP-scFv and tested its binding to platelets. After generating a protein of approximately 35 kDa (Supplemental Figure IIIA), our in vitro flow cytometry experiments demonstrated that EMP alone did not bind to either resting or activated platelets, regardless of the temperature used in the experiments (Supplemental Figure IIIB). Furthermore, flow cytometry demonstrated that EMP-scFv did not activate platelets, as the fusion protein had no effect on binding of PAC-1 (Supplemental Figure IVA) or P-selectin expression (Supplemental Figure IVB).

Temperature-Dependent Binding of EMP-scFv to Platelet Aggregates Under Flow Conditions and Temperature-Dependent Inhibition of Platelet Aggregation by EMP-scFv

To demonstrate that the fusion construct EMP-scFv is targeted to platelet aggregates, flow chamber experiments were performed. Hypothermic temperatures (22°C) caused binding of EMP-scFv to platelet aggregates, but normothermic temperatures (37°C) did not (Figure 5A). As a functional evaluation of platelets, effective inhibition of ADP-driven platelet aggregation by EMP-scFv at 22°C but not at 37°C was demonstrated using a 96-well plate-based light transmission aggregometry approach (Figure 5B). To further assess the effect of EMP-scFv on platelet aggregation, aggregate formation was directly visualized in microscopy. EMP-scFv inhibited aggregation of activated human platelets at 22°C but not at 37°C (Figure 5C).

Temperature-Specific In Vivo Antithrombotic Effects of EMP-scFv

As a final proof of the newly described strategy of a temperature-dependent drug, we performed in vivo experiments in mouse thrombosis models. As a first step, flow cytometry using mouse whole blood was performed to show that EMP-scFv bound to activated murine platelets at 22°C but not at 37°C (Supplemental Figure VA). EMP-scFv did not bind to resting mouse platelets at either 22°C or 37°C (Supplemental Figure VA). Furthermore, flow cytometry demonstrated a significant decrease in fibrinogen binding to activated mouse platelets at 22°C in the presence of EMP-scFv, whereas the fusion protein EMP-scFv had no effect on the binding of fibrinogen to mouse activated platelets in the experiments conducted at 37°C (Supplemental Figure VB).

To demonstrate the advantages of temperature-dependent, activation-specific GPIIb/IIIa blockade by EMP-scFv on arterial thrombosis in vivo, we chose a ferric chloride–induced mouse carotid artery thrombosis model. The EMP-scFv significantly prolonged the occlusion time in mice injected with the fusion protein at 28°C compared with the control group, showing a platelet-inhibitory effect similar to that observed with eptifibatide (Figure 6A). In contrast to this, at 37°C EMP-scFv was not active and did not prolong the arterial occlusion time (Figure 6A).

Lastly, we examined the temperature-dependent antithrombotic effects of EMP-scFv in a model of jugular venous thrombosis. The percentage of initial blood flow showed significantly higher levels of blood flow 15 minutes after the injury in mice injected with EMP-scFv at 28°C, compared with the control group and those experiments conducted at 37°C (Figure 6B). The effects on the jugular venous blood flow of EMP-scFv at 28°C are comparable to the clinically used GPIIb/IIIa blocker eptifibatide (Figure 6B).

Discussion

In this study, we investigated whether the fusion of an EMP to an activation-specific GPIIb/IIIa-blocking scFv enabled selective hypothermia-induced antiplatelet therapy. To achieve this, we fused EMP to the anti-GPIIb/IIIa-scFv, expressed this fusion construct in bacteria, and purified the protein. To illustrate how the β-spiral conformation of EMP may prevent the scFv from inhibiting GPIIb/IIIa at 37°C, we propose a structural model of the EMP-scFv fusion. This model indicates that the β-spiral EMP obstructs the scFv from binding to and in turn inhibiting GPIIb/IIIa. Below the conformational-transition temperature, ie, at 22°C, EMP is reported to adopt a random unstructured conformation.34 The random EMP conformation would not efficiently preclude the scFv from interacting with and inhibiting GPIIb/IIIa. The EMP-anti-GPIIb/IIIa-scFv fusion protein proved to be thermally responsive, as it demonstrated significant affinity for activated platelets at 22°C and 32°C but not at 37°C. The binding of fibrinogen to activated platelets was totally abolished in the presence of EMP-anti-GPIIb/IIIa-scFv at 22°C and 32°C, but it was not influenced at 37°C. We were also able to show that EMP-scFv prevented aggregation of activated platelets at 22°C but not when the temperature was increased to 37°C. In addition, our in vivo studies further demonstrated the antithrombotic efficacy of EMP-scFv, as the development of a thrombus in carotid arteries and jugular veins of mice treated with EMP-scFv was inhibited at 28°C, whereas no inhibition was observed at 37°C. This indicates that EMP-driven conformational change allows for temperature-dependent binding of scFv and hypothermia-specific inhibition of activated GPIIb/IIIa receptors on platelets. This was further confirmed by demonstrating the inabil-
Mild (31°C to 34°C), moderate (25°C to 30°C), and deep hypothermia (<20°C) are routinely used in cardiac surgery during extracorporeal circulation and especially for deep hypothermic circulatory arrest. Furthermore, hypothermia has also been used to lower patients’ body temperature during long-lasting neurosurgical procedures, and more recently it has been successfully applied to improve the recovery of patients after out-of-hospital cardiac arrest. However, in vitro and in vivo experiments have shown that hypothermic temperatures can induce platelet activation and lead to a rise in microvascular thrombus formation via platelet activation, resulting in fibrinogen binding to GPIIb/IIIa receptor. Furthermore, studies have shown that 33% to 88% of patients undergoing open-heart surgery experience some degree of postoperative neurological impairment and cognitive dysfunction. Hypothermia-induced platelet aggregation has also been shown to be closely related to cognitive decline after coronary artery bypass surgery. Neurological dysfunction after hypothermia exposure may therefore be caused by impaired perfusion of the microvasculature caused by hypothermia-induced platelet aggregates. In addition, hypothermia has been reported to cause microinfarctions of the liver caused by sequestered platelets, as well as thrombus formation in the pancreatic microcirculation leading to acute pancreatitis. Therefore, to inhibit platelet aggregation, preserve platelet function, and to avoid hemostatic dysfunction and platelet-mediated thrombosis during hypothermia, it is highly desirable to prevent hypothermia-associated platelet activation and platelet loss. At hypothermia, our EMP-scFv fusion protein was a highly potent blocker of the activated form of the GPIIb/IIIa receptor. Our findings demonstrated that platelet fibrinogen binding and aggregation were significantly inhibited by the EMP-scFv at 22°C but not at 37°C. These temperature-dependent antiaggregation effects of EMP-scFv are further supported by our flow chamber experiments, which showed that hypothermic temperatures induced the binding of EMP-scFv to platelet aggregates under physiological flow conditions. Furthermore, we demonstrated that

Figure 5. Binding of EMP-scFv to platelet aggregates and inhibition of platelet aggregate formation by EMP-scFv. A, Microscopic images demonstrate that hypothermic temperature induced the binding of fluorescence-labeled EMP-scFv to platelet aggregates under flow conditions. Typical examples of 4 experiments are given. B, Light transmission aggregometry demonstrates the ability of EMP-scFv to inhibit aggregation of platelets treated with ADP at 22°C but not at 37°C. The results represent the mean ± SD of 4 independent experiments. C, Microscopic images show that platelet aggregation was inhibited by EMP-scFv at 22°C but not at 37°C, whereas the addition of abciximab inhibited platelet aggregation at both temperatures. Typical examples of 3 independent experiments are given.
However, as the temperature is increased, the EMP segment in a loose, fully expanded state, referred to as a random coil.52 It is evident that the temperature-dependent binding of EMP-scFv to activated platelets at hypothermic conditions, EMP-scFv did not dissociate from the cells following the increase in temperature. By doing so, the fusion protein prevented the exposure of the unblocked active form of GPIIb/IIIa, thus inhibiting binding of fibrinogen and thrombus formation on rewarming. Therefore, the major advantage of the newly described temperature-steered GPIIb/IIIa inhibitor is its potential to protect platelets and prevent thrombosis during clinical settings in which hypothermia is used but its potential to protect platelets and prevent thrombosis.

Conformation-unspecific binding of GPIIb/IIIa inhibitors may have 2 major disadvantages. It has been postulated that their binding of EMP-scFv to platelets, as well as its ability to inhibit fibrinogen binding and ADP-driven platelet aggregation, was completely omitted. The in silico structural analysis of the EMP-scFv fusion protein supports this hypothesis. The RXD motif in the CDR3 region of the heavy chain, which constitutes the binding site of the anti-GPIIb/IIIa antibody,31 is masked by the β-spiral structure of the EMP and thus not accessible for binding to GPIIb/IIIa at 37°C. Therefore, this further confirms the ability of a temperature-responsive protein, such as EMP, to exert a novel control over an scFv function during hypothermic conditions.

Although the thermal transition profile of EMP-scFv revealed a transition temperature of 28°C, the fusion protein was still capable of binding to activated platelets and significantly inhibiting the binding of fibrinogen at 32°C. This is most likely because at 32°C, the EMP-scFv has not completely adopted the β-turn structure, suggesting that the RXD motif in the CDR3 region has not been completely blocked by EMP and is thus still capable of binding to activated form of GPIIb/IIIa. Previous studies have shown that the drop in protein concentration, as well as the properties of the accompanying fusion protein, can result in the increase of the real transition temperature.35,55 Therefore, because the concentration of EMP-scFv used in our flow cytometry experiments was no more than one fifth that applied in the CD studies, it is not unusual to see the binding of EMP-scFv to the activated platelets at 32°C, which is 5°C above its transition temperature determined by a CD assay. Drug activity at this temperature broadens the potential use of the newly described temperature-controlled strategy for the increasingly widely used mild therapeutic hypothermia to increase the survival rate and improve neurological outcome in patients after out-of-hospital cardiac arrest.46–48,56–58

The limited clinical data available suggest that pharmacological platelet inhibition during CABG with aspirin or clopidogrel protect patients from ischemic events.59,60 Furthermore, administration of GPIIb/IIIa blockers, such as eptifibatide and tirofiban, in the preoperative period of CABG has been reported to decrease the incidence of perioperative myocardial infarction and platelet loss, as well as the need for minor transfusions during CABG.26 Also, biochemical markers show less activation of the hemostatic and inflammatory system.61 However, despite these initial promising reports about the effects of GPIIb/IIIa blockers during cardiac surgery,26,62,63 the currently available agents, particularly abciximab, are too long-acting to provide controllable platelet inhibition that is restricted to the time period of the actual surgical intervention and thus are feared to result in bleeding complications after the surgical procedure.59,60,64–67 Overall, because of the dichotomy of antithrombotic (antiischemic) and antithrombotic effects of the currently clinically available antiplatelet drugs, strong evidence for the overall benefit of platelet inhibition in CABG has not been demonstrated. However, reversible antiplatelet compounds have been suggested to represent an ideal approach for platelet protection in patients undergoing CABG, providing support for the drug development strategy outlined in this study.60

Conformation-unspecific binding of GPIIb/IIIa inhibitors may
conformation-unspecific, ligand-mimetic binding can cause paradoxical platelet activation.69 Furthermore, the inhibitory effect of the conformation-unspecific blockade of all GPIIb/IIIa receptors on all circulating platelets is seen as a major reason for bleeding problems. In contrast, selective activation-specific blockade of GPIIb/IIIa by the scFv described promises a major benefit in terms of bleeding problems. In contrast, selective activation-specific blockade of all GPIIb/IIIa receptors on all circulating platelets is seen as a major reason for the prevention of thrombosis during surgical procedures performed under hypothermic conditions. This novel pharmacological strategy promises to provide antithrombotic protection during surgery, to conserve platelets against hypothermia-induced activation and to ensure fully functional platelets for intact hemostasis on rewarminv of the patient. This is the first description of a broadly usable pharmacological strategy, which uses temperature to fully control drug activity.

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Disclosures
None.

References
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Supplemental Figure I. Temperature-dependent inhibition of PAC-1 binding to activated platelets by EMP-scFv at 22°C, but not at 37°C. Histograms on the left represent FITC-labeled PAC-1 mAb binding to non-activated platelets (black lines, non-colored histograms) and activated platelets (black lines, blue histograms). Histograms in the middle and the right represent PAC-1 binding to activated platelets in the presence (red lines, non-colored histograms) and absence of EMP-scFv (black lines, blue histograms).
Supplemental Figure II. EMP-scFv binding and blocking properties at 32°C. A, Bar graphs and representative histograms represent binding of EMP-scFv to activated human platelets at 32°C [four independent experiments, mean ± SD, **p<0.01, (blue histograms represent the median fluorescence of EMP-scFv binding to resting platelets, while binding of the fusion protein to activated platelets is represented by red histograms)]. FITC-labeled PAC-1 mAb was used to confirm the ADP-dependent platelet activation (green histograms represent the median fluorescence of PAC-1 binding to resting platelets, while binding of the antibody to the activated platelets is represented by red histograms). B, Bar graphs and representative histograms represent the temperature-dependent blocking of GPIIb/IIIa at 32°C. Four conditions were tested examining inhibition of fibrinogen binding: in the absence of additive (no addition), in the presence of 10 µg/mL abciximab, and in the presence of 15 µg/mL and 50 EMP-scFv [four independent experiments, mean ± SD, **p<0.01, ***p<0.001, (red histograms represent the median fluorescence of polyclonal rabbit anti-human fibrinogen/FITC antibody binding to activated platelets, while blue histograms represent the effects of either abciximab or EMP-scFv on fibrinogen binding to activated platelets)].
Supplemental Figure III. Binding of EMP to human platelets. **A**, SDS-PAGE showing EMP at approximately 35 kDa after purification. **B**, Bar graphs represent temperature-dependent binding of EMP to activated platelets at different temperatures. No binding of the protein was detected regardless of the temperature used in the experiment. FITC-labeled PAC-1 mAb was used to confirm the ADP-dependent platelet activation. The results represent the mean ±SD of four independent experiments.
Supplemental Figure IV. *Effects of EMP-svFv on platelet activation.* Bar graphs demonstrating that EMP-scFv had no effects on PAC-1 binding (A) or P-selectin expression (B). The results represent the mean ±SD of four independent experiments.
Supplemental Figure V. Temperature-specific binding and blocking properties of EMP-scFv on mouse platelets. A, Bar graphs and representative histograms represent binding of EMP-scFv to activated mouse platelets at 22°C, but not 37°C [four independent experiments, mean ± SD, **p<0.01, (blue histograms represent the median fluorescence of EMP-scFv binding to resting platelets, while binding of the fusion protein to activated platelets is represented by red histograms)]. FITC-labeled anti-CD62P antibody was used to confirm the ADP-dependent platelet activation (red histograms represent the median fluorescence of anti-CD62P binding to resting platelets, while binding of the antibody to the activated platelets is represented by green histograms). B, Histograms represent the blocking of mouse GPIIb/IIIa at 22°C, but not 37°C. Three conditions were tested examining inhibition of fibrinogen binding: in the presence of 10 µg/mL eptifibatide, and in the presence of 15 µg/mL and 50 EMP-scFv (red histograms represent the median fluorescence of FITC-conjugated rabbit anti-fibrinogen polyclonal antibody binding to activated platelets, while blue histograms represent the effects of either eptifibatide or EMP-scFv on fibrinogen binding to activated platelets.
Supplemental Methods

Generation of the EMP-scFv Fusion Construct

Single-stranded oligonucleotides encoding the forward and reverse strands of monomer, (IPAVG)$_5$ (ATT CCG GCT GTT GGT ATC CCA GCT GTT GGT ATC CCA GCT GTT GGC ATT CCG GCT GTA GGT ATC CCG GCA GTG GGC) were chemically synthesized (Sigma Genosys, Inc.) with BamHI and HindIII overhangs and annealed to generate double-stranded oligonucleotides. The double stranded DNA cassettes were purified by agarose gel electrophoresis (4% GTG NuSieve agarose, 1X TBE buffer), phosphorylated and inserted into pZErO-1 cloning vector. Plasmid containing monomer DNA was propagated in the *E. coli* strain Top 10F’ and the inserts were screened and verified by DNA sequencing. Monomeric genes encoding (IPAVG)$_5$ were sequentially digested with restriction enzymes, BbsI and BsmBI, respectively, and isolated by agarose gel electrophoresis (4% GTG NuSieve agarose). The purified DNA monomers were concatamerized in a head-to-tail method via T4 DNA ligase. This multimerization of DNA monomers afforded to produce various sizes of multimers. A clone encoding sixteen repeats of the EMP monomer was isolated from insertion of multimer mixtures into the BsmBI site of the original plasmid containing the monomer DNA.

In order to clone the EMP gene into pET39b(+) expression vector, a cloning vector was modified such that the polylinker could accommodate the multimer at the BbsI sites and add a short linker sequence of GGGGS to the 3’ end of the multimer. EMP gene of the appropriate size in pZErO-1 was liberated by restriction digestion with BbsI and BsmBI, respectively, and inserted into the internal two BbsI sites of a polylinker in pZErO-2. A recombinant clone encoding sixteen repeats of monomer and short linker sequence was cloned into the pET39b(+) vector using NcoI restriction sites, and then sub-cloned in pHOG-21 bacterial expression vector already containing single-chain antibody (scFv) cDNA incorporating His(6)-tag sequence, resulting in a final fusion construct outlined in Figure 1. The pHOG-21 expression vector contains a pelB leader peptide which allows for periplasmic localization of the recombinant protein within the bacteria. This bacterial expression vector is also characterized by the
presence of a His(6)-tag for the Ni$^{2+}$ which facilitates purification and detection of recombinant proteins. It also contains an ampicillin resistance gene used for selection.

The cloning strategy resulting in the fusion protein consisting of N-terminal EMP and C-terminal scFv motif was chosen in order to generate the fusion construct exhibiting a tT that would be suitable for the current study, as previous EMP studies have shown that the orientation of the fused proteins modulates the direction of change in tT of EMP.\textsuperscript{2,3} The fusion of EMP to the C-terminus of the fusion partner significantly elevates the tT (>50°C), whereas the fusion of EMP to the N-terminus results in a decreased tT (<35°C) of the fusion construct.

**Expression of EMP-scFv Construct in E. coli and Protein Purification**

*E. coli* (TG1) cells were transformed with the pHOG-21 plasmid described above and plated on an agar plate containing 100 μg/mL ampicillin. Starter culture was established by inoculating a single colony into 10 mL of LB media containing 100 μg/mL ampicillin and growing it overnight in a 37°C incubator at 200 rpm. The following day, starter culture was transferred into 1 L of fresh LB containing 100 μg/mL ampicillin and the cultures shaken at 220 rpm for approximately 4 – 6 hours until an OD (600 nm) of ~ 0.6 was reached, followed by the addition of IPTG to a final concentration of 1 mM for induction of scFv production and incubated at 37°C with 200 rpm for 6-8 hours. For purification of insoluble protein from whole cell extract, bacteria were harvested by centrifugation at 5000 rpm for 15 min at 4°C. Pelleted bacteria were resuspended in 5 mL of cold 1X BugBuster® (Novagen) solution/g pellet and incubated for 15 min at room temperature with gentle shaking. After an additional centrifugation at 15 000 rpm for 20 min at 4°C, the supernatant was discarded and inclusion bodies containing insoluble protein resuspended in 15 mL solution containing 1:10 dilution of ice cold BugBuster®. The resuspended inclusion bodies were incubated at room temperature for 15 minutes with gentle shaking, before centrifuging at 5000 rpm for 15 min at 4°C. The wash step was repeated 4 more times, followed by the resuspension of the inclusion body pellet in buffer B (8 M urea, 100 mM NaH$_2$PO$_4$, 100 mM Tris HCl, pH 8). The insoluble fraction was purified by passing through a purification column previously layered with 600 μL of Ni$^{2+}$-Agarose beads.
(Qiagen). The fraction was applied through the same column 4 times to ensure the maximal binding of His(6)-tagged proteins. The column was washed with 7 mL of buffer B, 7 mL of buffer C (8 M urea, 100 mM NaH2PO4, 100 mM Tris HCl, pH 6.3) and 7 mL of buffer D (8 M urea, 100 mM NaH2PO4, 100 mM Tris HCl, pH 5.9). Finally, EMP-scFv fusion proteins were eluted with buffer D (8 M urea, 100 mM NaH2PO4, 100 mM Tris HCl, pH 4.5) in approximately 8-10 separate fractions of 600 µL.

The proteins were refolded using 1 L re-folding (RF) buffer (100 mM NaH2PO4, 10 mM Tris-HCl, pH 7.4) at 4°C with gentle stirring, where the concentration of urea was reduced every two hours from 6 M, 4 M and to 2 M, before finally incubating the protein samples in 4 L of 1x PBS overnight at 4°C. The concentration of dialyzed fractions was determined using BCA assay, and the integrity of the protein assessed via SDS-PAGE and Western Blotting under reducing conditions. Proteins were transferred onto an Immobilon P membrane (Millipore Corporation) for immunoblotting. After blocking the membrane overnight with phosphate buffered saline containing 0.2 % Tween20 (PBS-Tween) and 1 % BSA, a HRP-labeled anti-His(6)-antibody (Roche) was added (dilution 1:2000) and incubated for 2 hours at room temperature. The membrane was washed several times with PBS-Tween buffer. Visualization of peroxidase activity was achieved by addition of SuperSignal® Chemiluminescent Substrate (Pierce) on a ChemiDoc XRS® (BioRad).

**Circular Dichroism (CD) and Thermal Transition Profile Monitoring**

Circular dichroism (CD) spectra were recorded on a Jasco J-810 spectropolarimeter equipped with a PFD-425S Peltier temperature control unit in 0.1 cm sealed quartz cells at concentrations of 7.5 µM (EMP), 8.4 µM (scFv) and 4.1 µM (EMP-scFv) in 50 mM sodium phosphate buffer, pH 7.4. Temperature/wavelength CD-scans were performed within the temperature range from 5°C to 35°C with equilibration for 10 min at each temperature. Spectra were obtained from 260 to 190 nm at a resolution of 0.5 nm and at a scanning speed of 50 nm/min. The CD curves represented the average of five measurements and were smoothed using the means-movement method on the interval analysis of the spectral manager program. CD data are reported as mean residue ellipticity ([θ], deg cm² dmol⁻¹). Thermal transition curves were plotted by the
mean residue ellipticity as a function of temperature for the disappearance of the random coil and the appearance of the β-turn structure.

**In Silico Structural Analysis of EMP-scFv Fusion Peptide**

A model of the single-chain antibody, was generated by automated protein structure homology modelling using the publicly available SWISS-MODEL server, a fully automated protein structure homology-modelling server (http://swissmodel.expasy.org/) accessible via the ExPASy web server, or from the program DeepView (Swiss Pdb-Viewer). This model used Protein Data Bank entry 3bn9D as a template, which shows 77.2% sequence identity to the antibody. The model is missing the last 21 residues of the C-terminus. This structure was analysed using PROCHECK and minimized using the Tripos force field and Tripos partial atomic charges with 10000 iterations (all other parameters were set to the default values), until the iteration reached a gradient consensus.

A β-spiral model of EMP was constructed with the Biopolymer module in Sybyl8.1 (http://triplos.com/) using the parameters described by Luan and Urry (1992) and minimized using the procedure described above for the scFv model. The angles of the Pro (Φ -70, Ψ 120) and Gly (Φ 80, Ψ 0) residues were manually altered to conform with previous molecular mechanics studies of elastin and minimized under the Tripos force field, using the same procedure as that carried out for the scFv model, for 5089 iterations before a gradient consensus was reached. The scFv and EMP protein models were then docked together using RosettaDock (http://rosettadock.graylab.jhu.edu/).

**Flow Cytometry**

Human blood was collected by venipuncture with a 21-gauge butterfly needle from healthy volunteers and anticoagulated with citric acid. Platelet-rich plasma (PRP) was obtained by centrifugation (GS-6R centrifuge, Beckman Coulter) at 100 x g at room temperature for 10 min.

Mouse blood was collected by intracardiac puncture with a 27-gauge needle from C57BL/6 mice and anticoagulated with non-fractionated heparin (20 U/mL). A volume of 50 μl was washed with 1 mL
modified Tyrode’s buffer (150 mM NaCl, 2.5 mM KCl, 1.2 mM NaHCO₃, 0.1 % BSA, 0.1 % Glucose) and centrifuged at 1200 x g for 6 min. The supernatant was discarded and the pellet was resuspended in 1 mL modified Tyrode’s buffer containing 2 mM MgCl₂ and 2 mM CaCl₂.

Human PRP was diluted 1/50 in modified Tyrode’s buffer containing 2 mM MgCl₂ and 2 mM CaCl₂. Platelets were either activated by addition of 20 μM ADP, or non activated, followed by the incubation with 15 and 50 μg/mL EMP-scFv fusion protein or EMP at 22°C, 32°C and at 37°C for 15 min. Mouse platelets were either pre-activated for 10 min by addition of 0.1 U/mL thrombin (Enzyme Research Laboratories), or non activated, before the incubation with 15 and 50 μg/mL of EMP-scFv fusion protein at 22°C, 32°C or 37°C for 15 min. In both, human and mouse samples, EMP-scFv was detected via 15 min in-dark incubation with secondary antibody (Penta-His Alexa Fluor 488, Qiagen) directed against the His(6)-tag of the single-chain antibody.

Fibrinogen binding to human activated platelets in the presence of EMP-scFv was determined with a polyclonal rabbit anti-human fibrinogen FITC-labeled antibody (DakoCytomation), while the effects of EMP-scFv on fibrinogen binding to mouse platelets were determined using FITC-conjugated rabbit anti-fibrinogen polyclonal antibody (Cemfret). Blocking experiments with human platelets included 10 μg/mL abciximab (Eli Lilly) as a positive control, while 10 μg/mL of eptifibatide (Millenium) was used in the same experiments conducted with mouse platelets. Fluorescence detection was performed as described above. Samples were measured in a FACSCalibur® flow cytometer (Becton Dickinson), after fixation with 1x CellFIX® (Becton Dickinson).

In re-warming experiments, activated and non-activated diluted human PRP was incubated with 15 and 50 μg/mL of EMP-scFv for 15 minutes at 22°C, followed by a 15 minutes in dark incubation with secondary Penta-His Alexa Fluor 488 antibody. Half of the reaction was then fixed with 1x Cell FIX, while the other half was incubated at 37°C for either 15 or 30 minutes, before being fixed and measured in FACS.

Effects of EMP-scFv on platelet activation were addressed by incubating human diluted PRP with 15 and 50 μg/mL of EMP-scFv at 22°C for 15 minutes, followed by the addition of either PAC-1 or anti-
CD62P antibodies (both from Becton Dickinson) and in-dark incubation for 15 minutes. The reactions were fixed and levels of PAC-1 binding and P-Selectin expression analyzed in FACS.

**Platelet Aggregometry and Targeting of EMP-scFv to Platelet Aggregates under Flow Conditions at Different Temperatures**

Light transmission aggregometry was performed using a Biorad Benchmark plate reader, following a previously established 96-well plate method\(^8\). In brief, after incubation of human PRP with EMP-scFv (15 μg/mL), vehicle (PBS) or the GPIIb/IIIa blocker abciximab (10 μg/mL) (Reopro®, Eli Lilly, Indianapolis, U.S.A) for 10 minutes at different temperatures, the aggregation was induced by the addition of 2 or 20 μM ADP with vigorous stirring in a 96-well plate. The absorbance was determined at 595 nm every 15 seconds for 16 minutes between vigorous shaking at 22°C and 37°C.

For the microscopic analysis of platelet aggregates at different temperatures, non-diluted human PRP was placed onto a glass slide and ADP was added to a final concentration of 20 μM, in the presence and absence of EMP-scFv (50 μg/mL). Abciximab was used as a positive control at a concentration of 10 μg/mL. The experiments were conducted at 22°C and 37°C. Phase contrast microscopy images were taken using an Olympus CKX41 microscope.

Temperature-dependent binding of EMP-scFv to platelet aggregates was analyzed using a flow chamber system. Vitrotubes rectangular capillaries (0.20x 2.0 mm) were washed with 70% ethanol, dried, and coated overnight with 10 μg/ml of collagen (Collagen Reagent Horm Nycomed). Liquid was entered into the capillary via capillary drag. Capillaries were blocked for 1 hour with 1% BSA and then washed with PBS. The capillary was connected on the one end to a reservoir and on the other end to a Harvard medical pump by tubing. Before connecting the capillary, all tubes were flushed with PBS in order to avoid air bubbles entering the capillary. PRP was then drawn into the capillary and numerous platelet aggregates were formed within 10 minutes. The capillary was then washed with PBS via the pump. In the meantime, the EMP-scFv (50μg/mL) was incubated with Penta-His Alexa Fluor 488 (Qiagen) antibody for 10 minutes in a final volume of 1 mL, and then drawn into the capillary containing platelet aggregates.
at a flow rate of 60s\(^{-1}\). The capillary was imaged with a 20x objective, after which DIC and fluorescent images were captured using Olympus IX81 microscope with XM10 camera. The experiments were conducted at 22\(^\circ\)C and 37\(^\circ\)C.

**In Vivo Functional Evaluation of Anti-thrombotic Efficacy of EMP-scFv at Different Temperatures**

C57BL/6 mice weighing from 20-25 g were used. Care and use of laboratory animals followed the national guidelines and were approved by the institutional animal care and ethics committees. Mice were anesthetized with isoflurane, following an intra-peritoneal injection with ketamine (100 mg/kg BW) and xylazine (20 mg/kg BW). The body core temperature was monitored with a digital thermometer, and controlled at either 28\(^\circ\)C or 37\(^\circ\)C with a thermal pad (Temperature Controller TR-200, Fine Science Tools Inc.). Reducing the body temperature to 28\(^\circ\)C was achieved by a gradual drop, at a rate of 0.3-0.4 \(^\circ\)C/min. The mice on the thermal pad were placed under a dissecting microscope (SZ61, Olympus). An incision of the skin was made directly on the top of trachea, and vessels used for experiment were bluntly isolated from surrounding tissues. A catheter was cannulated into the right-side of the Jugular vein, and all administrations were injected into the blood flow via the cannula. Either EMP-scFv, (4.25 \(\mu\)g/g body weight), eptifibatide (0.18 \(\mu\)g/g) or saline (5 \(\mu\)l/g body weight) were administered 5 min prior to induction of the injury on the vessel. Thrombosis on either carotid artery or jugular vein was induced by applying a piece of filter paper (1x2 mm, GB003, Schleicher & Schuell) saturated with ferric chloride (10% solution) (Sigma) beneath the isolated vessel and removed after three minutes. A piece of Parafilm was laid to prevent the injury on surrounding tissue. After rinsing with normal saline, a nano-Doppler flow probe (0.5 VB, Transonic) was then positioned over the vessel and the blood flow rate (ml/min) was measured by a transit-time perivascular flow-meter (TS420, Transonic Systems Inc.). The carotid artery thrombotic occlusion was considered to occur when flow decreased to 0.0 \(\pm\) 0.2 mL/min, a range corresponding to the accuracy of the system as specified by the manufacturer. Jugular vein blood flow was recorded 15 minutes after induction of injury in mice pre-treated with EMP-scFv, eptifibatide or saline. The blood flow
recorded at one minute was considered as the initial blood flow rate. The percentage of blood flow rate at 15 minutes over the initial flow rate was compared among different administration groups.

**Statistical Analysis**

Data are presented as mean ± SD for the indicated number of mice and blood donors. The statistical comparisons were made by 2-way ANOVA, followed by Newmann-Keuls test, and differences were considered to be statistically significant at $p < 0.01 (**)$.

References


