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Novel Antiplatelet Drug Revacept (Dimeric Glycoprotein VI-Fc) Specifically and Efficiently Inhibited Collagen-Induced Platelet Aggregation Without Affecting General Hemostasis in Humans

Martin Ungerer, MD; Kai Rosport, MSc; Andreas Bültmann, PhD; Richard Piechatzek, MD; Kerstin Uhland, PhD; Peter Schlieper, MD; Meinrad Gawaz, MD; Götz Münch, MD

Background—Blocking of glycoprotein VI–dependent pathways by interfering in vascular collagen sites is commonly seen as an attractive target for an antiplatelet therapy of acute atherosclerotic diseases such as myocardial infarction or stroke. Revacept (soluble dimeric glycoprotein VI-Fc fusion protein) has been shown to reduce platelet adhesion by blocking vascular collagen in plaques or erosion and to be safe in preclinical studies. A dose-escalating clinical phase I study was performed to assess the safety, tolerability, pharmacokinetics, and pharmacodynamics of Revacept in humans.

Methods and Results—In a first-in-humans study, 30 healthy men received a single intravenous administration of 10, 20, 40, 80, or 160 mg Revacept. The serum concentration–time courses of each dosage of Revacept showed a narrow variation and a concentration and time dependence. Revacept did not significantly affect the bleeding time. Collagen-induced platelet aggregation was dose-dependently inhibited up to 48 hours at lower doses and for 7 days after higher dose levels. In contrast, ADP- or thrombin receptor activating peptide–dependent platelet aggregation remained unaltered. There were no relevant drug-related adverse events or drug-related changes in laboratory parameters (biochemistry, hematology, and coagulation parameters). There were no drug-related changes in blood pressure, pulse rate, or ECG parameters (including 24-hour Holter monitoring). No anti-Revacept antibodies were detected.

Conclusion—This phase I study demonstrated that Revacept is a safe and well-tolerated new antiplatelet compound with a clear dose-dependent pharmacokinetic profile with specific, dose-related inhibition of platelet aggregation despite completely unaltered general hemostasis.

Clinical Trial Registration—URL: www.clinicaltrials.gov. Unique identifier: NCT 01042964. URL: eudract.ema.europa.eu. Identifier: 2005–004656-12. (*Circulation*. 2011;123:1891-1899.)

Key Words: glycoprotein VI ■ platelet adhesion ■ platelet aggregation ■ platelet aggregation inhibitors ■ stroke

Platelets play a crucial role in the development and complications of coronary heart disease. Platelet adhesion after plaque rupture or erosion of the atherosclerotic artery leads to the subsequent steps of thrombus formation and ischemic complications in coronary heart disease.¹ Similarly, stroke can be caused by the occlusion or embolization of a thrombus from the major brain-supplying blood vessels.

Clinical Perspective on p 1899

In recent years, a number of high-performing antiplatelet compounds have been developed that block thrombus formation and have been introduced successfully into the

treatment of patients with myocardial infarction. Current antiplatelet agents have been shown to reduce ischemic events, but often at the expense of increased bleeding.^{2–5} These findings provide an important basis to study additional compounds with the potential to inhibit platelet adhesion and activation and collagen aggregation without affecting general hemostasis.

In contrast to existing drugs, which inhibit all platelets and therefore incur markedly increased risk of bleeding, compounds that specifically interfere in the collagen-induced activation of platelets hold promise for reduced risk of complications. Revacept (PR-15, dimeric glycoprotein [GP] VI-Fc) has been found to have the same efficacy

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as known antiplatelet drugs, but did not incur any risk of bleeding complications in animal studies.⁶ It is directed against collagen in endothelial lesions, and thereby scavenges the pivotal ligand for the activation of crucial platelet receptors.⁷ Revacept potently inhibits collagen-induced aggregation of human platelets *ex vivo*,⁸ but not thrombin- or ADP-induced aggregation. Exposure of human blood to human plaque material from carotid endarterectomy *ex vivo* in a flow chamber resulted in relevant local platelet aggregation that was clearly inhibited by anti-GPVI (Glycoprotein VI) antibodies or GPVI downregulation⁹ and Revacept.¹⁰ In contrast to anti-GPVI antibodies,¹¹ however, Revacept neither induced downregulation of the platelet receptor nor reduced platelet count.

Here, we present the results of a clinical phase I study (EudraCT 2005–004656-12, NCT 01042964) that aimed to investigate the pharmacokinetic, pharmacodynamic, and safety parameters of Revacept in healthy male volunteers.

Methods

Revacept is a fusion protein consisting of the Fc part of human IgG1, a short hinge region derived of the same protein, a specific linker sequence, and the extracellular part of human GPVI.⁶ This fully human fusion protein was expressed in Chinese hamster ovary cells. It was obtained as a covalently linked dimer of a molecular mass of 150 kDa as determined under nonreducing conditions by SDS-PAGE.

Revacept was produced by fermentation at Orpegen GmbH (now GlycoTope-Biotech GmbH), Heidelberg, Germany, and manufactured at Evotec GmbH, Hamburg, Germany, and Glasgow, Scotland, according to good manufacturing practice guidelines.

Study Design

The trial, An Open Label Dose Escalating Safety and Pharmacokinetic Study of an Acute Intravenous Administration of PR-15, an Inhibitor of Platelet Adhesion, in Different Doses in Healthy Male Volunteers, was carried out at Medifacts GmbH (now ABX-CRO GmbH), Görlitz, Saxonia, Germany, from March 2006 through October 2007. It was granted approval by the local ethics board of Saxonia in 2004 (No. JU-EK-AMG-35/2004), and was amended in 2005 and 2006. The conception of the trial, drafting of the study protocol, supervision of the study, and interpretation of the analytic results were provided entirely by the predecessor company to Corimmun GmbH, Procorde GmbH, Martinsried, Germany, and its management and scientists and Dr Schlieper, Greifengberg, Germany, who served as a clinical research officer. After obtaining regulatory permission to start the trial, Procorde GmbH was merged with Trigen AG, Martinsried. The study was independently monitored by Dr W. Gielsdorf, Ulm, Germany, and regular audits were carried out by Dr P. Schlieper.

Initially, the trial was a randomized study. After the inclusion of the first 6 subjects, the trial was opened with approval of the ethics committee to an open-label dose-escalation study. The trial was conducted in accordance with International Conference on Harmonization guidelines for good clinical practice issued in July 1996 in accordance with the Declaration of Helsinki and with local laws and regulations. The trial was registered in the Eudra CT (Eudra CT 2005–004656-12) and clinical.trials.gov (NCT 01042964) databases.

Study Objectives

The primary objective was to evaluate the safety and tolerability of Revacept after a single intravenous dose by clinical assessment, laboratory parameters (hematology, blood chemistry and urinalysis, serology test, drug tests in urine), measurement of bleeding time, and evaluation of any possible antibody titers against Revacept. The online-only Data Supplement provides details on materials and methods.

The secondary objectives were to evaluate following the pharmacokinetic parameters: area under the serum concentration-time curve from time 0 to the last experimental time point (t) with a detectable drug concentration (C_t) that is greater than the limit of quantification [$AUC_{(0-t)}$]; area under the serum concentration-time curve from time 0 to infinity [$AUC_{(0-\infty)}$], calculated as $AUC_{(0-t)} + C_t/k_{el}$, where k_{el} is the elimination rate constant estimated from a log linear regression plot of the terminal elimination phase comprising at least the last 3 observed serum concentrations; maximum observed concentration in plasma (C_{max}); time to C_{max} (t_{max}); terminal half-life of the drug in serum ($t_{1/2}$) calculated as $t_{1/2} = \ln 2/k_{el}$; total clearance of drug after intravenous administration, defined as the ratio of the dose level and $AUC_{(0-\infty)}$; mean residence time, given as $AUMC_{(0-\infty)}/AUC_{(0-\infty)}$, where $AUMC_{(0-\infty)}$ is the area under the first moment curve; and steady-state volume of distribution. For further details, please see the online-only Data Supplement.

Inclusion and Exclusion Criteria

Subjects meeting all of the following criteria were eligible for study participation: healthy, nonsmoking white men between 18 and 35 years of age who were normotensive (systolic blood pressure ≤ 140 mm Hg and diastolic blood pressure ≤ 90 mm Hg) with a body weight ranging from 75 to 85 kg. All subjects were tested and shown negative in assays searching for anti-HIV antibodies, HBs antigens, and hepatitis C virus. All gave written informed consent to study participation. The online-only Data Supplement provides further details.

Subjects

Thirty healthy men participated after providing written informed consent. Before inclusion in the study, the general health of the volunteers was ascertained by a detailed medical history, physical examination, ECG recording, and clinical chemistry tests. All subjects concluded the study according to the study protocol.

Drug Administration

The study medication was provided in solution in glass vials containing 40 mg that was prepared by the investigating physician to the respective dose in an infusion system and infused intravenously over 20 minutes. The trial medication was warmed to body temperature (37°C) just before dosing. Doses of Revacept were given as an infusion of 10, 20, 40, 80, or 160 mg to cohorts of 6 healthy men each. The study was initially started as randomized trial. Because the serum half-life of Revacept turned out to be longer than anticipated, the study protocol was modified by an addendum submitted to a vote by ethics boards. Thereafter, the study was conducted as an open-label dose-escalation study starting with 10 mg. After completion of each dose group, a safety board discussed all findings before the next higher dose was administered to the subjects.

Adverse Events

Adverse events were monitored during the complete course of the study through investigator inquiries, spontaneous reports, and clinical evaluations, such as physical examinations, vital sign measurements, ECG (including 24-hour Holter monitoring), and clinical laboratory tests (eg, hematology, blood chemistry, coagulation, and urinalysis). For further details, please see the online-only Data Supplement.

Laboratory Parameters

In all subjects, drug safety was monitored by laboratory parameters before and after drug administration (see the online-only Data Supplement).

Additionally, on the evening of day 0, drug screening was carried out in urine. The Abuscreen test kit (Hoffmann-La Roche, Mannheim, Germany) was used to screen for amphetamines, methamphetamines, barbiturates, benzodiazepines, cocaine, opiates, tetrahydrocannabinol, and phencyclidine. An alcohol breath test was performed with a Dräger Alcotest 7410 PLUS. For further details, please see the online-only Data Supplement.

Table 1. Demographic Overview and Baseline Characteristics of Study Subjects

| Group/Dose Level | n | Age (years) | Weight (kg) | Height (cm) | BMI (kg/m ²) | Smoker | Medical History | Previous Medication |
|------------------|---|-------------|-------------|-------------|--------------------------|--------|-----------------|---------------------|
| 10 mg | 6 | 28.3±9.4 | 76.1±5.76 | 176.5±6.3 | 24.4±0.6 | 2 | 2 | 0 |
| 20 mg | 6 | 29.0±10.3 | 80.7±6.3 | 183.2±3.5 | 24.0±1.3 | 2 | 2 | 0 |
| 40 mg | 6 | 22.7±2.4 | 75.6±8.2 | 182.7±9.7 | 22.7±1.8 | 1 | 3 | 0 |
| 80 mg | 6 | 34.0±7.1 | 81.5±5.1 | 184.8±5.1 | 23.9±1.7 | 2 | 4 | 0 |
| 160 mg | 6 | 30.2±5.9 | 75.7±6.7 | 183.5±7.5 | 22.5±1.7 | 4 | 2 | 0 |

BMI indicates body mass index. A total of 30 white men (6 per dose group) were enrolled in the study. All participants completed the study according to the protocol. Values are mean±SD when appropriate.

Bleeding Time and Platelet Aggregation

Bleeding time was measured via ear lobe incision (before and 3 hours after dosing) according to the Duke method.

Pharmacodynamics of Revacept was evaluated with platelet aggregation assays. The aggregation of human platelets was determined from samples of platelet-rich plasma after stimulation with collagen, prepared from rabbit aorta, ranging from 0.45 to 2.81 $\mu\text{g/mL}$, ADP (20 $\mu\text{mol/L}$), or thrombin receptor activating peptide (25 $\mu\text{mol/L}$) for all samples in triplicate. The concentrations of thrombin receptor activating peptide and ADP were chosen in accordance with previous studies.^{12,13} Collagen-induced aggregation was titrated in each subject before drug administration to a minimum of 30% of the reference platelet-poor plasma sample, with adjusted collagen concentrations ranging from 0.45 to 2.81 $\mu\text{g/mL}$. This collagen concentration was documented individually for each subject and then used throughout the whole course of the study. The online-only Data Supplement provides further details.

Preparation of Collagen From Rabbit Aorta

Rabbit collagen was isolated from freshly prepared aortic vessel walls; rabbit aortas from 7 healthy male New Zealand White rabbits (age, 14 weeks; body weight, 3 kg; source, Asamhof, Kissing, Germany) were used for preparation of a single batch, which was then used during the whole study (No. 30–09-05; see the online-only Data Supplement). The stability of the frozen rabbit collagen was tested by analyzing its potency to stimulate platelet aggregation. The potency was unaltered over a period of 14 months.

Analysis of the Generation of Anti-Revacept Antibodies by ELISA

The generation of antidrug antibodies was excluded by subjecting serum samples derived from blood withdrawn 1, 22, and 43 days after drug administration by specific ELISA. The assays were performed at MDS Pharma (Allendorf, Switzerland) according to good laboratory practice principles. The online-only Data Supplement has additional details.

Determination of the Revacept Concentration in Human Serum by ELISA

For pharmacokinetic analyses, Revacept concentrations in human serum derived from blood withdrawals before and at distinct time points (5 minutes, 30 minutes, 1 hour, 4 hours, 12 hours, 24 hours, 72 hours, 120 hours, and 14 days) after drug administration were determined by ELISA. The analysis was performed by MDS Pharma Services (Allendorf, Switzerland) in accordance with the principles of good laboratory practice (see the online-only Data Supplement).

Statistical Methods

Data were presented by summary statistics as follows: number of observations, arithmetic mean, and SE or SEM as indicated. The normality assumption was tested by applying the Kolmogorov-Smirnov test to all samples. It was shown that all groups of data were normally distributed. Therefore, ANOVA followed by the Bonferroni analysis was used to test for significant differences between dosing groups and changes of aggregation values after Revacept

administration at different time points with repeated measures ANOVA. For log-normal-distributed pharmacokinetic parameters, geometric means and geometric coefficients of variation were determined. For each dose level, pharmacokinetic parameters were determined by means of descriptive statistics. The linear regression for the correlations between Revacept doses and AUC was calculated with Excel software according to the ordinary least-squares regression line algorithm.

All adverse events were tabulated in full detail with respect to type, onset, duration, severity, and drug relation. Vital signs (blood pressure, pulse rate), 12-lead ECG, safety laboratory data, and antibody titers were evaluated by means of descriptive statistics.

Results

A total of 30 white men were divided into groups 1 through 5 (n=6 each) and received 10, 20, 40, 80, or 160 mg Revacept, respectively. All enrolled subjects completed the trial according to the study protocol. The demographic data revealed no significant differences with regard to weight, height, and body mass index, so the 5 dose groups were comparable (see Table 1). Incidentally, the ages of the 40-mg and 80-mg dose groups differed slightly.

Pharmacokinetics

The pharmacokinetics of each dosage of Revacept is shown in Figure 1. It demonstrates a narrow variation and a concentration and time dependence. C_{max} and AUC increased

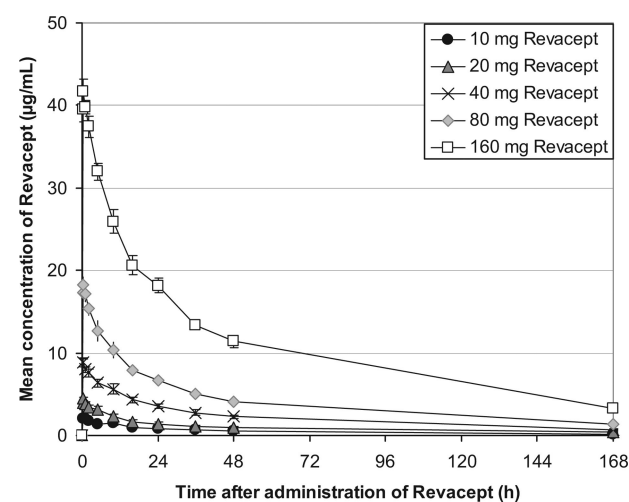


Figure 1. Pharmacokinetics of Revacept in human serum samples. Serum levels of Revacept before, during, and after a 20-minute intravenous infusion were determined by sandwich ELISA. Mean serum levels of each dose group (n=6) with SEM are plotted over time.

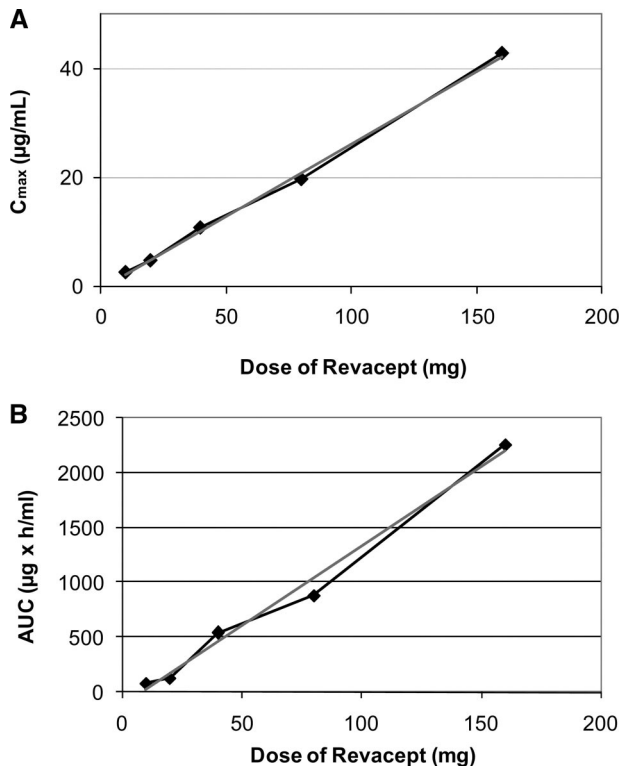


Figure 2. Correlation of pharmacokinetic parameters with the applied dose. Mean maximum observed concentration in plasma (C_{max} ; **A**) and mean area under the curve (AUC; **B**) are plotted over the respective doses of Revacept. The graphs show good linear correlations of pharmacokinetic parameters with the applied dose.

linearly with increasing dose (Figure 2). The t_{max} stayed almost constant (because of intravenous administration of the medication). The elimination was slow; Revacept could still be detected 3 days after administration of the 10-mg dose ($\approx 0.5 \mu\text{g/mL}$) and higher. After 2 weeks, $1 \mu\text{g/mL}$ could still be measured in the serum of those subjects who had received the 160-mg dose. The terminal half-life was lower in the 2 lower-dose groups than in the groups receiving 40, 80, and 160 mg, where a comparable half-life was determined. Correspondingly, the clearance was slow and the mean residence time high. The volume of distribution at steady state ranged between 7.8 ± 2.7 and 12.3 ± 2.8 L, suggesting that Revacept is confined primarily to the systemic circulation. All parameters are summarized in Table 2.

Table 2. Pharmacokinetic Parameters

| Group/Dose Level | AUC _(0-t) ($\mu\text{g} \times \text{h/mL}$) | AUC _(0-\infty) ($\mu\text{g} \times \text{h/mL}$) | C_{max} ($\mu\text{g/mL}$) | t_{max} (h) | $t_{1/2}$ (h) | k_{el} (1/h) | CL (mL/min) | MRT (h) | Vss (L) |
|------------------|---|--|--------------------------------|---------------|------------------|-------------------|----------------|------------------|----------------|
| 10 mg | 80.8 \pm 23.3 | 96.6 \pm 24.9 | 2.9 \pm 1.1 | 2.1 \pm 3.8 | 67.7 \pm 6.5 | 0.010 \pm 0.001 | 1.8 \pm 0.5 | 70.0 \pm 8.3 | 7.8 \pm 2.7 |
| 20 mg | 187.9 \pm 101.0 | 215.9 \pm 97.93 | 5.1 \pm 1.2 | 1.3 \pm 1.8 | 87.5 \pm 22.7 | 0.008 \pm 0.002 | 1.8 \pm 0.64 | 95.1 \pm 30.9 | 9.2 \pm 1.5 |
| 40 mg | 515.8 \pm 120.6 | 546.0 \pm 126.0 | 11.4 \pm 2.4 | 0.9 \pm 0.8 | 129.7 \pm 7.9 | 0.005 \pm 0.000 | 1.3 \pm 0.33 | 139.0 \pm 11.6 | 10.7 \pm 3.3 |
| 80 mg | 898.0 \pm 184.6 | 948.7 \pm 192.3 | 20.0 \pm 1.8 | 0.9 \pm 0.8 | 137.6 \pm 27.2 | 0.005 \pm 0.001 | 1.5 \pm 0.3 | 142.6 \pm 27.2 | 12.3 \pm 2.8 |
| 160 mg | 2330.3 \pm 245.3 | 2416.6 \pm 266.9 | 44.1 \pm 3.6 | 0.6 \pm 0.2 | 136.6 \pm 36.7 | 0.005 \pm 0.001 | 1.1 \pm 0.1 | 133.6 \pm 35.8 | 9.0 \pm 2.9 |

The pharmacokinetic parameters area under the serum concentration-time curve from time 0 to the last experimental time point [AUC_(0-t)], area under the first moment curve [AUC_(0-\infty)], maximum observed concentration in plasma (C_{max}), time to C_{max} (t_{max}), terminal half-life of the drug in serum ($t_{1/2}$), elimination rate constant (k_{el}), total clearance of drug after intravenous administration (CL), mean residence time (MRT), and steady-state volume of distribution (Vss) were calculated from serum concentrations of Revacept using noncompartmental procedures. Values are mean \pm SD for 6 volunteers per dose group.

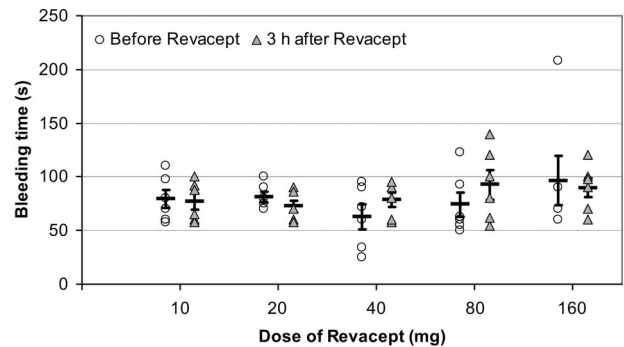


Figure 3. Effect of Revacept on the bleeding time. Bleeding times of all individuals of each dose group ($n=6$) as determined by an earlobe incision (the Duke method) before (\circ) and 3 hours after (\blacktriangle) administration of Revacept are shown. Means of each group are shown with SEM (black bars). No significant differences occurred between the dosing groups before or after Revacept administration.

General Hemostasis: Bleeding Time, Coagulation, and Platelet Count

Bleeding times as determined with the Duke method (earlobe incision) within each dose group were similar before and 3 hours after administration of Revacept. There were no significant differences between the 5 dose groups before and at 3 hours after Revacept infusion (Figure 3). We therefore concluded that Revacept did not have any significant effect on bleeding time.

There were no significant differences in platelet counts between the Revacept dose groups (Figure 4). Revacept also had no influence on general coagulation in the subjects' blood as assessed by activated partial thromboplastin time and international normalized ratio in the dosing groups before and 3 days after Revacept administration (Figure 5A and 5B).

Platelet Aggregation: Pharmacodynamics

Measurements of maximum aggregation showed that there was a strong and clearly significant time- and dose-dependent inhibition of collagen-induced aggregation after Revacept administration in all dose groups (Figure 6A). The effect started at least 2 hours after infusion of Revacept, and remained up to 24 hours after higher doses (40, 80, and 160 mg). In the 40-mg dose group, the collagen-induced aggregation returned to baseline on day 22 ($43.6 \pm 3.6\%$). In the 80- and 160-mg dose groups, aggregation returned to normal on day 14 ($44.7 \pm 9.2\%$ and $32.9 \pm 4.9\%$, respectively). Revacept

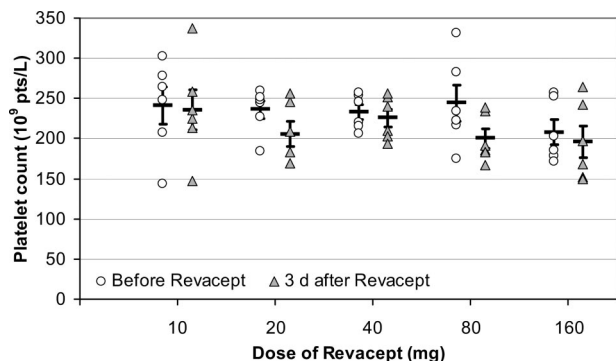


Figure 4. Effect of Revacept on platelet count. Platelet counts of all individuals of each dose group (n=6) as determined by routine hematology before (○) and 3 days after (▲) administration of Revacept are shown. Means of each group are shown with SEM (black bars). No significant differences occurred between the dosing groups before or after Revacept administration.

10 mg inhibited significantly weaker collagen-mediated aggregation compared with higher doses at 24 hours after drug application ($P<0.0001$). Moreover, 10 and 20 mg Revacept also inhibited significantly weaker collagen-mediated aggregation ($P<0.05$) 7 days after drug administration compared with 40 or 160 mg Revacept. Thus, Revacept shows a certain dose effect with prolonged inhibition of collagen-mediated platelet aggregation at

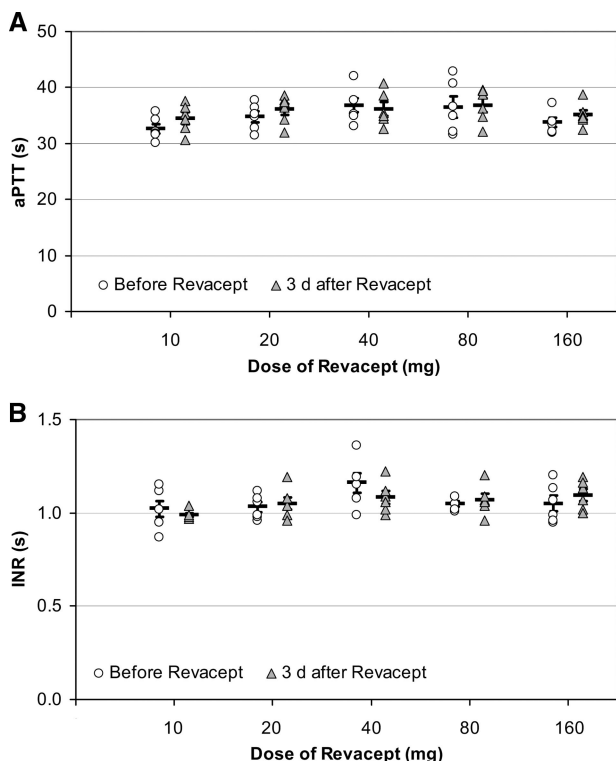


Figure 5. Effect of Revacept on blood coagulation. Activated partial thromboplastin time (aPTT; **A**) and international normalized ratio (INR; **B**) of all individuals of each dose group (n=6) as determined by routine hematology before (○) and 3 days after (▲) administration of Revacept are shown. Means of each group are shown with SEM (black bars). No significant differences occurred between the dosing groups before or after Revacept administration.

later time points after administration. In contrast, ADP- or thrombin receptor activating peptide-induced aggregation was not altered by administration of Revacept during the entire observation period (Figure 6B and 6C).

Safety Assessment

No serious adverse events were recorded during the complete duration of the study. There were 2 occurrences of mild to moderate intensity 4 and 20 days after the beginning of treatment. One subject showed symptoms of tonsillitis; the other subject exhibited symptoms of nasopharyngitis. The 2 subjects recovered, and the adverse events resolved without sequelae and were judged to be unrelated to treatment with the study drug.

There were some abnormal laboratory parameters (biochemistry and hematology) at screening, all of them not clinically relevant and evenly distributed among the different dosages. There was no trend toward a deterioration of any of these parameters in relation to Revacept. Systolic blood pressure values were all within the normal range. Four subjects (3 in the 20-mg group and 1 in the 160-mg group) showed abnormal changes in diastolic blood pressure at different time points. However, an effect of Revacept on diastolic blood pressure could not be derived from these results because 3 subjects showed a decrease and 1 subject showed an increase in diastolic blood pressure compared with screening values. No subject had a significant difference in diastolic blood pressure when data of measurements directly before and up to 48 hours after drug administration were compared.

Several subjects showed an abnormal change in pulse rate, defined as >20 bpm, after drug administration compared with pulse rate at screening. However, all of these subjects presented with differences in pulse rate directly before drug application compared with screening. There was no influence of Revacept on heart rate when pulse rates up to 48 hours after drug application were compared with those directly before drug application.

One subject in the 40-mg dose group had newly recognized incomplete right bundle-branch block in a 12-lead ECG 8 and 36 hours after drug administration. Two subjects had incomplete right bundle-branch block at screening and after drug administration. This finding was recorded as an abnormality by the investigators, but the pathological relevance of this finding is not clear and is a commonly seen variation in healthy young people. The other ECG parameters, such as P wave, PQ intervals, QRS complex, and QT intervals, were not significantly changed by the study medication.

The ECG measurements by 3-lead Holter monitoring showed no triplets, salvos, atrial fibrillation, or paced beats during the observational period. Couplets, ventricular tachycardia, and pauses were seen only once in different dose groups and only at least 15 or 22 hours after drug application. No bigeminy, trigeminy, or artifacts were recorded during the observational period. Therefore, we conclude that there was no proarrhythmic effect of the drug.

The assay for anti-Revacept antibodies was negative in all samples assayed (assay sensitivity, 0.32 μg/mL) 22 and 43 days after drug administration.

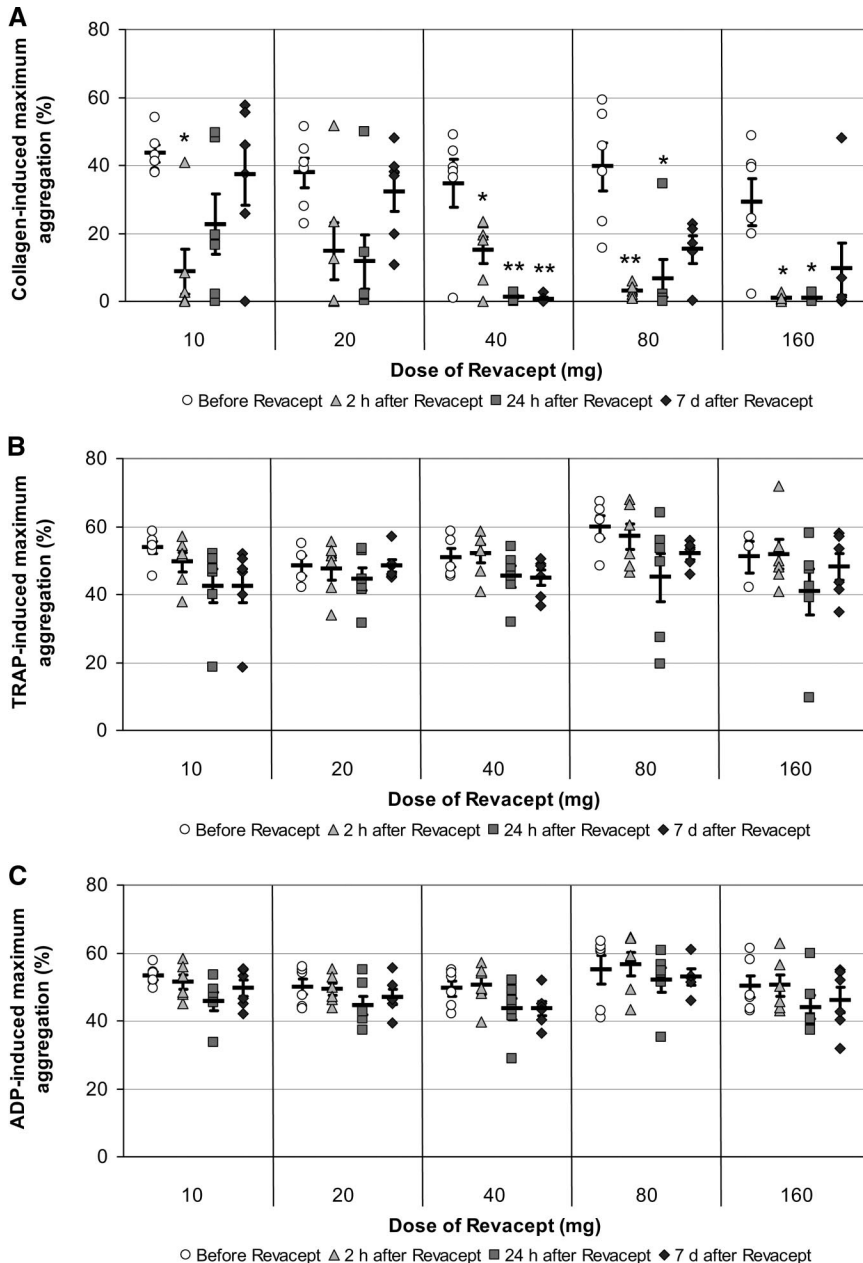


Figure 6. Effect of Revacept on platelet aggregation. Maximum platelet aggregation induced by collagen (**A**), thrombin receptor activating peptide (TRAP; **B**), and ADP (**C**) was determined in blood samples drawn from the study subjects of all dose groups (n=6) before and 2 hours, 24 hours, and 7 days after administration of Revacept. All individual results are demonstrated for the doses of Revacept from 10 to 160 mg per subject. Means are shown as black bars with SEM. Revacept reduced the collagen-induced platelet aggregation in a time- and dose-dependent manner, whereas TRAP- and ADP-induced platelet aggregation was not affected. *Statistical significance ($P \leq 0.05$) and **strong statistical significance ($P \leq 0.02$) when the maximum aggregation of each dose group at distinct time points after Revacept administration was compared with the corresponding aggregation at baseline.

Discussion

The clinical phase I study demonstrated that Revacept is a safe and well-tolerated new compound with a clear dose-dependent pharmacokinetic profile and a dose-related, highly specific pharmacodynamic action. Revacept had no effect on general hemostasis, as determined by measuring bleeding times, or on coagulation, as determined by activated partial thromboplastin time and international normalized ratio. No anti-Revacept antibodies were detected up to 6 weeks after administration. The serum concentration initially decreased very rapidly, with a long-lasting remaining low concentration of Revacept. Collagen-induced platelet aggregation was dose-dependently inhibited up to 7 days with prolonged inhibition of collagen-mediated platelet aggregation after higher dose levels. In contrast, ADP- or thrombin- (thrombin receptor activating peptide-) dependent platelet aggregation remained unaltered.

Despite progress in the understanding of the mechanisms of plaque-mediated arterial thrombosis, current antithrombotic concepts rely on the prevention of platelet aggregation by fibrinogen receptor antagonists, aspirin, and P_2Y_{12} antagonists. These concepts have the disadvantage of targeting the consequences, not the source, of the platelet activation and aggregation during acute vascular syndromes. Recent clinical data with intravenous fibrinogen receptor antagonists^{14,15} and novel antiplatelet drugs such as prasugrel¹⁵ showed that improvements in the efficacy of platelet inhibition and the prevention of ischemic complications are often achieved at the expense of increased bleeding complications. Therefore, our aim was to focus on optimal safety in the search for a potent and safe agent to protect patients from arterial thrombosis. Glycoprotein VI has been considered a pivotal protein involved in platelet adhesion and tethering and in promoting the initial signaling cascade that leads to thrombus formation

at sites of arterial injury or plaques (see elsewhere^{1,7} for a review). Glycoprotein VI has also been implicated in thrombus formation at human plaque material from carotid endarterectomy *ex vivo*,^{9,10} and GPVI-Fc/Revacept has been shown to bind to atherosclerotic plaques of humans and of different animal species.^{8,16} Moreover, in patients with acute coronary syndrome,^{17,18} transient ischemic attack, and stroke¹⁹ platelet GPVI receptor expression is increased. Increased concentrations of soluble GPVI were found in the plasma of patients with atrial fibrillation and acute coronary syndrome.²⁰ Thus, GPVI seems to be an indicator or even a mediator of platelet activation and thrombus formation in clinical settings with atherothrombosis. Interestingly, in patients with high GPVI expression levels, basal platelet activity was significantly increased despite dual antiplatelet therapy.¹⁸ Therefore, we aimed at inhibiting collagen as the most thrombogenic ligand of atherosclerotic plaques that initiates early stages of platelet activation to develop a safe antiplatelet agent to treat acute coronary syndrome, transient ischemic attack, and stroke. Revacept is the soluble, dimeric form of the GPVI receptor and binds to collagen and fibronectin at the atherosclerotic plaque (Figure 7A).^{6,16} The mechanism is illustrated in Figure 7B, which shows that Revacept, by binding to the atherosclerotic plaque, prevents platelet adhesion and consecutive thrombus formation at the site of vascular injury. Revacept thus prevents a very early step in acute vascular syndromes. Revacept was shown to inhibit local thrombus formation in a previous animal study.⁶ The efficacy was similar to that of anti-GPVI antibodies studied in the same model,²¹ which, however, induced markedly prolonged platelet inhibition as a result of internalization of GPVI and even platelet depletion in animals.²² In several subjects who were identified with idiopathic thrombocytopenic purpura caused by anti-GPVI autoantibodies, low platelet counts were observed, together with a mild bleeding disorder.^{23,24} In contrast, no platelet depletion occurred after administration of Revacept in either the short or long term. Moreover, the efficacy of Revacept is increased compared with other GPVI-Fc fusion proteins, one of which showed efficacy only at clearly higher concentrations.¹¹

The present *ex vivo* results from healthy volunteers after Revacept administration showed dose-dependent inhibition of collagen-mediated platelet aggregation, with almost complete inhibition for 24 hours with a single dose of 20 mg and for up to 7 days with higher doses. Aggregation was normalized in all patients at the end of the observation protocol. Other GPVI interventions such as anti-GPVI antibodies, however, led to a moderate prolongation of bleeding time^{21,22} both with and without downregulation of GPVI expression on platelets²⁵ with 1 exception.²⁶ The combination of anti-GPVI antibodies with aspirin severely compromised hemostasis in mice,²⁷ which did not occur when Revacept was added to the same dose of acetylsalicylic acid in mice (unpublished results). Revacept specifically inhibits collagen-mediated platelet adhesion and aggregation and has no effect on ADP- and thrombin/thrombin receptor activating peptide-mediated activation pathways. This shows another aspect of the specificity and

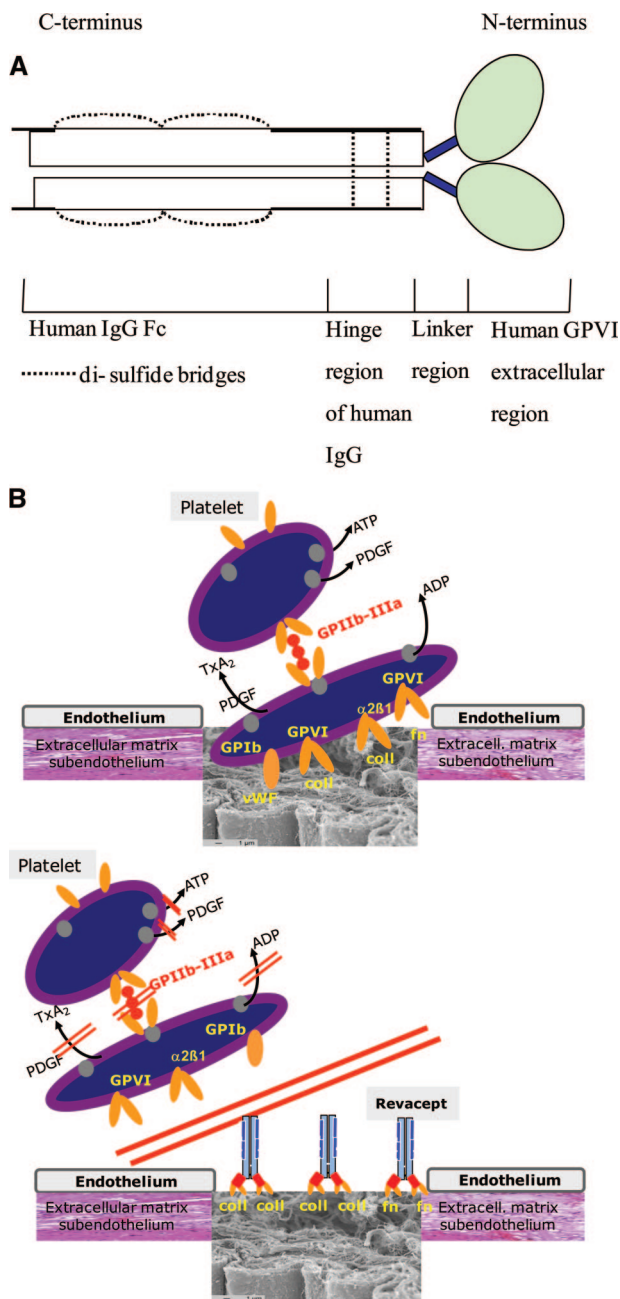


Figure 7. Revacept structure and mode of action. **A**, Schematic structure of Revacept as a dimeric Fc fusion protein with the IgG part and the extracellular domain of the human glycoprotein VI (GPVI) platelet receptor. **B**, Simplified model for platelet adhesion, aggregation, and activation in atherosclerotic vessels. Specific interaction of ligands in the endothelium and extracellular matrix of the subendothelium with various platelet receptors (**top**). Revacept binds to the ligands collagen (coll) and fibronectin (fn) in atherosclerotic stable or ruptured plaques. Revacept prevents the first steps of collagen-mediated platelet adhesion and the consecutive platelet aggregation, crosslinking, and platelet activation with consecutive release of procoagulant signals in acute arterial syndromes.

therefore safety of the drug and its potential superiority to anti-GPVI antibodies, which also showed an additional inhibition of thrombin responses in platelets.²⁸

There was a pharmacokinetic-pharmacodynamic relationship; ie, the Revacept plasma concentration suppressed maximum collagen-induced aggregation starting 2 hours after

drug administration that was significant 24 hours and 7 days after infusion at higher doses. The pharmacodynamic action persisted longer than anticipated from previous animal studies, which was probably due to the longer serum half-life of this fully human protein drug in men compared with that seen in the previous animal studies.

Therefore, the concept of lesion-directed inhibition of thrombus formation with Revacept seems a promising approach for the development of a safe and potent platelet inhibitor for the treatment of acute vascular syndromes. Moreover, long-term GPVI inhibition by Revacept seems to ameliorate atherosclerosis^{8,16} and has significant beneficial effects on the remodeling after catheter intervention in arteries.²⁹ Thus, a potent and safe anti-GPVI intervention with Revacept would impose beneficial effects on a broad range of atherothrombotic complications beyond the short-term application, which are currently only incompletely addressed by existing drugs in clinical routine.

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Disclosures

Drs Ungerer and Götz Münch are employed by Corimmun and own shares of Corimmun. Dr Uhland owns phantom shares of Corimmun and is employed by Corimmun. Dr Gawaz is a cofounder of Corimmun, owns shares of Corimmun, and is a professor at the Cardiology Department of the University of Tübingen. He also has received honoraria from Lilly, Bristol-Myers Squibb, and Bayer-Schering and is a consultant for Bayer-Schering. Dr Pichatzek is employed by ABX-CRO. Dr Schlieper is employed by the CRO Mediceo. The other authors report no conflicts.

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CLINICAL PERSPECTIVE

Treating atherothrombosis with Revacept in patients with acute cerebral arterial syndromes or acute coronary syndromes is a novel concept. This study drug is a soluble form of the platelet glycoprotein VI receptor, and binds specifically to collagen structures of ruptured plaques. Therefore, the first steps of platelet adhesion and the consecutive platelet aggregation are prevented without affecting the general platelet function and hemostasis. Revacept would be the first drug to potently block platelet function without increasing bleeding complications in patients. Preclinical and phase I studies in healthy volunteers have proved the mode of action and safety of this study drug. Despite tremendous progress in the treatment of patients with acute coronary or cerebral syndromes, decreased thrombus formation and reduction of ischemic complications are often achieved at the expense of increased bleeding. Therefore, a potent drug that inhibits platelet activation but would not affect general hemostasis would pose a significant improvement for the treatment of patients with acute arterial syndromes. The problem of increased bleeding has often hampered therapeutic benefits in the prevention or reduction of ischemia by antiplatelet or other antithrombotic drugs, especially in patients with ischemic strokes. Efficacy studies in patients must prove that the expectations will hold true in the future to develop a safe and potent platelet inhibitory drug.

SUPPLEMENTAL MATERIAL:

Supplemental Methods:

Revacept is a fusion protein consisting of the Fc part of human immunoglobulin G1 (IgG1), a short hinge region derived of the same protein, a specific linker sequence, and the extracellular part of human glycoprotein VI (GPVI)⁶. This fully human fusion protein was expressed in CHO cells. It was obtained as a covalently linked dimer of a molecular mass of 150 kDa as determined under non-reducing conditions by SDS-PAGE.

Revacept was produced by fermentation at Orpegen GmbH (now Glycotope-Biotech GmbH), Heidelberg, Germany, and manufactured at Evotec GmbH, Hamburg and Glasgow, Scotland according to GMP guidelines.

Study design

The trial is an open label dose escalating safety and pharmacokinetic study of an acute intravenous administration of PR-15, an inhibitor of platelet adhesion, in different doses in healthy male volunteers. It was carried out at the site of Medifacts GmbH (now ABX-CRO GmbH), Görlitz, Saxonia, Germany, from March 2006 through October 2007. It had been granted approval by the local ethics board of Saxonia in 2004 (# JU-EK-AMG-35/2004), and was amended in 2005 and 2006. The conception of the trial, drafting of the study protocol, supervision of the study and interpretation of the analytical results was entirely provided by the predecessor company to Corimmun GmbH, Procorde GmbH, Martinsried, Germany, and its management and scientists, and Dr. Schlieper, Greifenberg, Germany, who served as CRO. After having obtained regulatory permission to start the trial, Procorde GmbH was merged to Trigen AG, Martinsried. The study was independently monitored by Dr. Gielsdorf, Ulm, Germany, and regular audits were carried out by Dr. Schlieper.

The trial was conducted in accordance with International Conference on Harmonization (ICH) guidelines for Good Clinical Practice (GCP) issued in July 1996, in accordance with the Declaration of Helsinki, and with local laws and regulations.

The trial was registered at the Eudra CT data base (Eudra CT 2005-004656-12), and at the clinical.trials.gov data base (NCT 01042964).

Objectives of the study

The primary objective was to evaluate the safety and tolerability of Revacept after single IV dose by clinical assessment, laboratory parameters (hematology, blood chemistry and urinalysis, serology test, drug tests in urine), measurement of bleeding time, and evaluation of any possible antibody titers against Revacept. These safety parameters included medical history, ECG (12-lead) and 24 hour holter ECG, blood pressure and pulse rate, and detection of adverse events.

The secondary objectives were to evaluate the pharmacokinetic parameters $AUC_{(0-t)}$ [$ng \cdot h/ml$] (area under the serum concentration-time curve from time 0 to the last experimental time point (t) with a detectable drug concentration (C_t) that is greater than the limit of quantification), $AUC_{0-\infty}$ [$ng \cdot h/ml$] (area under the serum concentration-time curve from time 0 to infinity, calculated as $[AUC_{(0-t)} + C_t/k_{el}]$), C_{max} [ng/ml] (maximum observed concentration in plasma), t_{max} [h] (time to C_{max}), $t_{1/2}$ [h] (terminal half-life of the drug in serum, calculated as $t_{1/2} = \ln 2/k_{el}$), k_{el} [1/h] (elimination rate constant, estimated from a log linear regression plot of the terminal elimination phase comprising at least the last three observed serum concentrations), CL [ml/min] (total clearance of drug after intravenous administration, defined as the ratio of the dose level D and $AUC_{(0-\infty)}$), MRT [h] (mean residence time, given as $AUMC_{(0-\infty)}$ (area under the first moment curve)/ $AUC_{(0-\infty)}$), V_{ss} [L] (steady state volume of distribution). Pharmacokinetic parameters were determined on the

basis of serum concentrations of Revacept determined by ELISA (see below) from serum samples derived from blood withdrawals before and until 7 days after Revacept administration. For safety reasons, in selected subjects blood samplings were continued until the drug was completely eliminated from the serum compartment.

Inclusion and Exclusion criteria

Subjects meeting all of the following criteria were eligible for study participation: Healthy, non-smoking male Caucasians between 18 and 35 years of age, who were normotensive (systolic BP \leq 140 mmHg and diastolic BP \leq 90 mmHg), with a body weight ranging from 75 to 85 kg. All subjects were tested and shown negative in assays searching for anti-HIV antibodies, HBs antigens (HBsAg), and hepatitis C virus (HCV). They all gave Informed written Consent to study participation.

Subjects were not included for any of the following reasons: Subjects who were taking any prescription medication within the 14 days before study start or any non-prescription medication, especially, anti-platelet drugs, within the last seven days prior to the administration of trial medication. Exclusion also resulted from history of hypersensitivity, contraindication or serious adverse reaction to inhibitors of platelet aggregation or hypersensitivity to related drugs (cross-allergy). Subjects were also excluded if there was a history or clinical evidence of any cardiac, cardio- or cerebrovascular, hepatic, renal, pulmonary, endocrine, neurological, infectious, gastrointestinal, haematological, oncological, or psychiatric disease, emotional problems, or any other clinically relevant condition, physical findings, ECG- or laboratory test abnormality, which would pose a significant risk for the subject, invalidate the Informed Consent, or limit the ability of the subject to comply with study requirements. Exclusion criteria also included any history of alcohol and/or drug abuse

(verified by drug screening), and subjects who smoked more than 5 cigarettes per day and/or were unable to abstain from smoking during the entire in-house period.

Subjects

30 healthy males participated after providing written Informed Consent. Before inclusion in the study, the general health of the volunteers was ascertained by a detailed medical history, physical examination, ECG recording, and clinical chemistry tests. All subjects concluded the study according to the study protocol.

Drug administration

The study medication was provided in solution in glass vials containing 40 mg each, which were prepared by the investigating physician to the respective dose in an infusion system and infused intravenously over 20 minutes. The trial medication was warmed up to body temperature (37°C) just before dosing. Doses of Revacept were given as infusion of 10, 20, 40, 80, or 160 mg to cohorts of 6 healthy male volunteers each. The study was initially started as randomized trial. Since the serum half-life of Revacept turned out to be longer than anticipated, the study protocol was modified by an addendum which was submitted to Ethical Board's vote. Thereafter the study was conducted as an open label dose-escalation study starting with 10 mg. After completion of each dose group, a safety board discussed all findings before the next higher dose was administered to the subjects.

Adverse Events

Adverse events (AEs) were monitored during the complete course of the study through investigator inquiries, spontaneous reports, and clinical evaluations such as physical

examinations, vital sign measurements, ECG (including 24 hour holter monitoring), and clinical laboratory tests (e.g., haematology, blood chemistry, coagulation, and urinalysis).

Laboratory parameters

In all subjects, drug safety was monitored by the following laboratory parameters before and after drug administration: Biochemistry: ALAT/SGPT, albumin, alkaline phosphatase, ASAT/SGOT, blood urea nitrogen, calcium, chloride, creatine phosphokinase, γ -GT, creatinine, lactate dehydrogenase, glucose, sodium, potassium, total bilirubin, total protein, total cholesterol, triglycerides, and uric acid. Haematology: Haemoglobin, platelet count, haematocrit, white blood cell count (WBC), and red blood cell count (RBC). In case WBC was $< 3.500/\mu\text{l}$ or $> 11.000/\mu\text{l}$ a differential blood count, i.e., neutrophils, lymphocytes, eosinophils, basophils, and monocytes, was performed. Coagulation: Activated partial thromboplastin time (aPTT) and international normalized ratio (INR). All tests were carried out with standard systems by Medizinisches Labor Ostsachsen (Görlitz, Germany). Urinalysis was carried out by using the Combur¹⁰-dipstick method (Hoffmann-La Roche, Mannheim, Germany): Protein, glucose, red blood cells, and pH. Urinary sediment was always performed if an abnormal result was found for any parameter of the dipstick test.

Additionally, on the evening of Day 0 drug screening was carried out in urine, where amphetamines, methamphetamines, barbiturates, benzodiazepines, cocaine, opiates, tetrahydrocannabinol, and phencyclidine (PCP) were screened with the AbuscreenTM test kit (Hoffmann-La Roche, Mannheim, Germany). An alcohol breath test was performed using a Dräger AlcotestTM 7410 δ PLUSö.

Bleeding times and platelet aggregation

Bleeding time was measured via ear lobe incision (pre-dose and 3 hours after dosing)

according to Duke's method. In brief, after a small incision in the ear lobe was done with a standard lancet, blood was wiped away every 30 seconds with a filter paper until bleeding ceased. Bleeding from the incision was visually observed and bleeding time counted in seconds until cessation.

Pharmacodynamics of Revacept was evaluated by using platelet aggregation assays. The aggregation of human platelets was determined from samples of platelet rich plasma (PRP) after stimulation with collagen, prepared from rabbit aorta ranging from 0.45 to 2.81 $\mu\text{g/ml}$, ADP (20 μM), and TRAP (thrombin receptor activating peptide, 25 μM) respectively, for all samples in triplicates.

PRP was generated from citrated blood samples by low speed centrifugation (210 x g for 10 min at room temperature using low acceleration without brake). Blood samples were initially taken from each subject before and 2 hours, 24 hours, and 7 days after intravenous administration of Revacept. Since the serum half-life of Revacept turned out to be longer than anticipated, the study protocol was modified by an addendum which was submitted to Ethical Board's vote. From then on, for safety reasons, in case of persisting effect of the remaining Revacept on collagen-induced platelet aggregation, measurements of platelet aggregation were continued at weekly intervals until collagen-induced aggregation was restored.

All measurements were carried out within 2.5 hours after blood sampling. To prepare platelet poor plasma (PPP), platelets were removed from PRP by centrifugation at 5,000 x g for 10 min.

The aggregation of platelets was determined with an optical aggregometer (Chrono-Log-Aggregometer, Chrono-Log Corporation, Haverton, PA). This is a fixed wavelength spectrophotometer with two sample chambers heated to 37°C, containing a magnetic stirrer which mimics the shear conditions during blood flow. The transmission of infra red light through two cuvettes, one containing PRP as sample and one containing platelet poor plasma

(PPP) from the same subject as reference, was measured at baseline and until 7 min after the administration of the agonist, i.e. 20 μ M ADP, 25 μ M TRAP, or a pre-defined, subject-specific amount of collagen prepared from rabbit aorta ranging from 0.45 to 2.81 μ g/ml. Signals were transferred to a computer with Aggrolink software (Chrono-Log Corporation, Haverton, PA), where the transmission value of the non-stimulated PRP sample is set to 0 % aggregation; the reference value of the PPP sample equals 100 % aggregation, and the transmission data obtained until 7 min after agonist administration were used to calculate maximum aggregation for each data set.

Collagen-induced aggregation was titrated in each subject before drug administration to a minimum of 30 % of the reference PPP sample with adjusted collagen concentrations ranging from 0.45 to 2.81 μ g/ml. This collagen concentration was documented individually for each subject and then used throughout the whole course of the study.

Preparation of collagen from rabbit aorta

Rabbit collagen was isolated from freshly prepared aortic vessel walls: Rabbit aortae from 7 healthy male New Zealand White rabbits (age 14 weeks, body weight 3 kg, source Asamhof, Kissing, Germany) were used for preparation of a single batch, which was then used during the whole study (# 30-09-05). Aortae were frozen and crushed in liquid nitrogen and homogenates were solved in medium (0.25 M sucrose, 10 mM HEPES, pH 7, containing 2 mM EDTA, 2 mM EGTA, 1 mM phenyl-methylsulfonyl fluoride (PMSF) centrifuged and suspended in 10 volumes of extraction buffer (0.275 M NaCl, 50 mM Tris-HCl, pH 8, 1 mM PMSF) to produce a final NaCl concentration of 0.25 M, and incubated for 4-5 hours at 4°C with constant magnetic stirring. The homogenate was centrifuged for 30 min at 4,770 x g at 4°C, the pellet suspended in 10 volumes of extraction buffer and incubated overnight at 4°C with constant magnetic stirring. The NaCl extract was separated from the insoluble material by two centrifugation steps at 4,770 x g for 30 min and 15 min, respectively, at 4°C. The

supernatant was then filtered through a Falcon cell strainer, 40 μm (Becton Dickinson, #352340), and then through a Pre-separation filter (Miltenyi Biotec # 130-041-407). The final collagen solution was aliquoted at a concentration of 0.018 mg/ml and stored at 620°C in extraction buffer until use.

The stability of the frozen rabbit collagen was tested by analysing its potency to stimulate platelet aggregation. The potency was unaltered over a period of 14 months.

Analysis of the generation of anti-Revacept antibodies by ELISA

The generation of anti-drug antibodies was excluded by subjecting serum samples derived from blood withdrawn 1, 22, and 43 days after drug administration by specific ELISA. The assays were performed at MDS Pharma (Allendorf, Switzerland) according to Good Laboratory Practice (GLP) principles. Wells of a 96-well micro titre plate (Maxisorp, NUNC) were coated with 1 $\mu\text{g/ml}$ Revacept in coating buffer over night at 5°C . Wells were washed with PBS containing 0.05 % (v/v) Tween 20 (PBS-T), blocked for 1 hour at 24°C , then washed before addition of 50 μl serum samples pre-diluted 1:10 in PBS-T containing 1 % BSA (PBS-T/BSA) and 50 μl recombinant GPVI-Fc that had been biotinylated with 2 mg/ml D-Biotinyl- ϵ -aminocaproic acid-N-hydroxysuccimide ester in DMSO (24 ng/ml in PBS-T/BSA). Each plate routinely included negative control samples (NC, human serum pool) and positive control samples (PC, serum pool, spiked with 150 ng/ml affinity purified rabbit polyclonal anti-GPVI antibody (Charles River) in quadruplicates, and quality control samples (QC, serum pool spiked with 75 ng/ml of the anti-GPVI antibody) in duplicates. During the subsequent 2 h incubation at 24°C , anti-Revacept antibodies present in PC, QC, and possibly also in the test samples, respectively, bound to Revacept coated onto the microtiter plate as well as to Revacept-biotin. The wells were then washed and 50 ng/ml streptavidin-poly horseradish peroxidase conjugate (RDI-PHRP 80-SA2, Research Diagnostic Inc.) was added.

After 1 hour and a further washing step, the bound conjugate was visualised by the addition of freshly prepared TMB substrate solution (0.5 mM 3,3',5,5'-tetramethylbenzidine in 30 mM calcium citrate buffer). The colour intensity was directly related to the amount of anti-Revacept antibodies in the sample. Samples with $OD \leq OD_{cut-off}$ were defined to be negative for anti-drug antibodies. The $OD_{cut-off}$ was determined for each plate using the following formula:

$OD_{cut-off} = \text{mean } OD_{NC(\text{plate I})} + K * \text{mean } OD_{PC(\text{plate I})}$, where $\text{mean } OD_{NC(\text{plate I})}$ is the mean OD value of the NC on plate I, $\text{mean } OD_{PC(\text{plate I})}$ is the mean OD value of the PC on plate I, and K is a constant for anti-Revacept antibodies in human serum that had been determined to be 0.018 in the related GLP validation study on the basis of 34 individual blank human serum samples, presumed to be negative for anti-Revacept antibodies.

Determination of the Revacept concentration in human serum by ELISA

For pharmacokinetic analyses, Revacept concentrations in human serum derived from blood withdrawals before and at distinct time points (5 min, 30 min, 1 h, 4 h, 12 h, 24 h, 72 h, 120 h, and 14 days) after drug administration were determined by ELISA. The analysis was performed by MDS Pharma Services (Allendorf, Switzerland) in accordance with the principles of GLP. Wells of a 96-well micro titre plate (F8 Maxisorp, NUNC) were coated with 10 µg/ml proprietary rat monoclonal antibody 1A5, specific for Revacept in coating buffer (carbonate-bicarbonate buffer, pH 9.4) over night at 5°C. Wells were washed with PBS-T, blocked with blocking buffer (SuperBlock®, Pierce) for 1 hour at 24°C, then washed before addition of samples that were pre-diluted × 1:1000 in PBS-T/BSA. Each plate routinely included standards consisting of Revacept (0 to 2000 pg/ml, final concentration) and QC samples. After 2 hours at 24°C, wells were washed and the proprietary primary detection antibody 4C9-Dig, a digoxigenin-labelled mouse monoclonal anti-GPVI antibody recognizing another epitope than 1A5, added at 1 µg/ml in PBST/BSA. After 1 h and another washing step, the secondary detecting antibody, poly horseradish peroxidase conjugated anti-

digoxigenin antibody (Roche), was added (1:3000 dilution of stock at 50U/ml in PBS-T/BSA) for 1 hour, then after further washes, conjugate bound in the complex was visualised as described above for the anti Revacept antibody ELISA. After 5 to 15 min, the enzymatic reaction was stopped by addition of 1 M sulfuric acid and the intensity of the resulting colour determined by using a microtiter-plate photometer (Vmax, Molecular Devices) at a wave length of 450 nm with a reference wave length of 650 nm. The concentrations were then determined on the basis of the standard curve fitted with the four parameter logistic curve fitting model provided by SOFTmax®PRO v4.7.1 software.