The extrinsic coagulation pathway in coronary artery disease and endotoxemia

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Citation for published version (APA):

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Chapter 4

Recombinant nematode anticoagulant protein c2, an inhibitor of the tissue factor/factor VIIa complex, in patients undergoing elective coronary angioplasty

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Abstract

Objectives
This study investigated the safety and pharmacodynamics of escalating doses of recombinant Nematode Anticoagulant Protein c2 (rNAPc2) in patients undergoing elective coronary angioplasty.

Background
Recombinant NAPc2 is a potent inhibitor of the tissue factor/factor VIIa complex that has the potential to reduce the risk of thrombotic complications during acute coronary syndromes and catheter based interventions.

Methods
In a randomized, double blind, dose-escalation, multi-center trial, 154 patients received placebo or rNAPc2 at doses of 3.5, 5.0, 7.5, and 10.0 µg/kg as a single subcutaneous administration 2-6 hours before angioplasty. All patients received aspirin, unfractionated heparin (activated clotting time >250 seconds) during angioplasty, and clopidogrel in case of stent implantation.

Results
The incidence of minor bleeding for the doses 3.5 to 7.5 µg/kg were comparable to placebo (6.7%), while an incidence of 26.9% was observed at the 10.0 µg/kg dose level (p<0.01). Four episodes of major bleeding occurred in the 5.0 µg/kg (n=3) and 7.5 µg/kg (n=1) dose groups. The bleeding episodes in the three patients in the 5.0 µg/kg dose group occurred concurrently with the administration of a glycoprotein (GP) IIb/IIIa receptor antagonist. The administration of a GP IIb/IIIa antagonist to one patient, each in the 3.5 and 7.5 µg/kg dose groups, was uneventful. Systemic thrombin generation, as measured by prothrombin fragment 1+2 (F1+2), was suppressed in all rNAPc2 dose groups to levels below pretreatment values for at least 36 hours. In the placebo group a distinct increase of F1+2 levels was observed following cessation of heparin.

Conclusion
Inhibition of the tissue factor/factor VIIa complex with rNAPc2, at doses up to 7.5 µg/kg, in combination with aspirin, clopidogrel, and unfractionated heparin appears to be a safe and effective strategy to prevent thrombin generation during coronary angioplasty. This study supports further investigation of the safety and efficacy of rNAPc2 in patients with acute coronary syndromes.
**Introduction**

Tissue factor (TF) is a significant component of atherosclerotic plaques and is thought to be the primary trigger of thrombosis following plaque rupture in patients with unstable coronary syndromes. When exposed to blood, TF forms a high-affinity complex with circulating factor VIIa (TF-factor VIIa) that initiates the coagulation cascade through the activation of factors IX and X, leading to the formation of thrombin and subsequent thrombosis. Consequently, inhibitors of TF-factor VIIa have been evaluated for their potential to reduce coronary thrombosis in experimental studies.

Recombinant Nematode Anticoagulant Protein c2 (rNAPc2) is an 85 amino acid protein that was isolated from the hematophagic hookworm *Ancylostoma caninum*. It specifically inhibits the complex TF-factor VIIa by a unique mechanism that involves the formation of a high affinity complex with zymogen or activated factor X prior to formation of the quaternary inhibitory complex with TF-factor VIIa. The utilization of zymogen factor X as an inhibitory scaffold obviates the need of generating activated factor X to inhibit the TF/factor VIIa complex. Theoretically, this should result in rapid and efficient inhibition of TF-factor VIIa by rNAPc2 following a thrombogenic challenge. To this point, rNAPc2 has been shown to be very effective in reducing the incidence of deep venous thrombosis without hemostatic compromise when administered prophylactically in patients undergoing knee arthroplasty. In addition, the formation of a complex with factor X results in a biological half-life of rNAPc2 of >50 hours.

The importance of TF/factor VIIa in coronary thrombosis suggests that rNAPc2 could be effective for the treatment of patients suffering from acute coronary syndromes. As the majority of these patients undergo percutaneous coronary interventions (PCI), we first performed a phase II dose ranging study with 4 escalating dosages of rNAPc2 in patients undergoing elective coronary angioplasty, to evaluate the safety of the drug and its ability to inhibit thrombin generation.

**Methods**

**Patients**

Five centers in the Netherlands enrolled patients between 18 and 80 years, with a history of stable angina and scheduled for elective PCI of 1 or 2 lesions of >50% diameter stenosis. Exclusion criteria were: total occlusion, unstable angina or myocardial infarction within 14 days, recent history of surgery, trauma, cardiopulmonary
resuscitation, confirmed peptic ulcer, previous history of abnormal bleeding, uncontrolled hypertension, renal or hepatic insufficiency, and treatment with or intended use of oral antithrombotic agents other than aspirin and clopidogrel during study period. Planned use of glycoprotein (GP) IIb/IIIa receptor antagonists was excluded since there was no information available regarding the potential interaction of rNAPc2 with these agents.

The study protocol was conducted according to the International Conference on Harmonisation Good Clinical Practice Guidelines and approved by the institutional review boards. Written informed consent was obtained from all patients.

Study design
This study was designed as a randomized, placebo controlled, double-blind, multicenter study, for the evaluation of the safety of rNAPc2 on top of standard treatment during PCI. Patients were randomized to treatment with rNAPc2 or placebo in a ratio of 4:1, respectively, in each dose group. As a dose group consisted of approximately 30 patients, this resulted in ±24 patients per group treated with rNAPc2 and ±6 patients received placebo. All placebo treated patients of each dose group were clustered into one placebo group. Following randomization, patients received a single subcutaneous dose of rNAPc2 or placebo 2-6 hours prior to PCI. The dosing of rNAPc2 was such that maximal levels were reached during PCI. After access to the femoral artery, unfractionated heparin (UFH) was administered as intravenous bolus injections to reach an activated clotting time of >250 seconds throughout the intervention. Sheath (5 or 6 French) removal after PCI was performed as soon as an activated clotting time <180 seconds was reached. Patients were hospitalized for 36 hours after dosing, and follow-up consisted of a telephone interview at 48 hours and a clinical assessment at two weeks. The four doses of rNAPc2 tested sequentially were 3.5, 5.0, 7.5, and 10.0 µg/kg bodyweight. Each increase in dose was dependent on review by an independent Data Safety Monitoring Committee (DSMC). All patients received aspirin of at least 80 mg per day throughout the study. If stent implantation was performed, a loading dose of 300 mg clopidogrel was administered followed by 75 mg daily for at least 3 weeks. Patients receiving a stent in the initial 3.5 µg/kg rNAPc2 dose group, were given clopidogrel after sheath removal as an additional safety measure at the start of the trial. Following the successful completion of this dose group, the protocol was amended, allowing clopidogrel administration within three days prior to the intervention to reflect current practice. Subsequently, a second
3.5 μg/kg dose group was completed. This led to a total of six treatment groups, one placebo and five on rNAPc2 (3.5, 3.5, 5.0, 7.5, and 10.0 μg/kg).

**Blood collection and assays**

Six blood samples were taken from each patient during hospitalization, i.e. before the administration of study drug, after access to the femoral artery but before the angioplasty, at 2 and 8 hours after the last bolus administration of UFH, and at 24 and 36 hours after study drug administration. Blood was collected in citrated vacutainer tubes and immediately centrifuged at 4 °C. Plasma samples were stored at -70 °C until assayed.

Thrombin generation was quantified as prothrombin fragment 1+2 (F1+2) and thrombin-antithrombin (TAT) complexes using enzyme-linked immunosorbent assays for both measurements (ELISA, Behringwerke AG, Marburg, Germany). Normal values ranged from 0.4 to 1.1 nmol/l for F1+2 and from 1.0 to 4.1 μg/l for TAT complexes. For plasma concentrations between 0.4 and 5.0 nmol/l of F1+2 and between 2.0 and 60.0 μg/l of TAT complexes, the intra-assay coefficient of variations varied between 5.0%-7.5% and between 4.0%-6.0%, respectively. The plasma concentrations of rNAPc2 were also analyzed by ELISA (Corvas International, San Diego, California).

**Study outcomes and definitions**

The primary safety outcome of the study was the incidence of bleeding complications. A major bleeding episode was defined as a clinically overt bleeding resulting in death; retroperitoneal, intracranial, or critical internal organ bleeding; a hemoglobin drop of ≥3 g/dL; or the requirement of transfusion of ≥2 units of blood. A minor bleed was any clinically significant bleeding that did not qualify as major; for example epistaxis, ecchymosis, macroscopic hematuria, puncture site bleedings such as a groin hematoma measuring >100 cm², or any other located hematoma. Groin hematoma size was determined by estimation of the surface area.

A secondary pharmacodynamic outcome was the extent of systemic thrombin generation performed by serial measurements of plasma levels of F1+2 and TAT complexes.

Myocardial infarction (MI) was defined as a creatine kinase-MB concentration more than three times the upper limit of normal.

We introduced a new quantitative safety measurement for puncture site (surgical) hemostasis, femoral compression time (FCT), defined as the groin compression time.
(in minutes) between sheath removal from the femoral artery and achievement of complete hemostasis. This required manual groin compression just proximal of the puncture site by experienced personnel using a standardized method described in the protocol. Continuous manual compression was mandated for the first 10 minutes followed by release, and if hemostasis was complete, FCT was recorded as 10 minutes. If not, compression was re-instituted for periods of 3 minutes until complete hemostasis, which was defined as no discernable bleeding externally or internally by careful inspection for at least one minute.

**Statistical analysis**

Descriptive statistics included mean values and standard deviations (SD), or median values and interquartile ranges (IQR), for outcomes with or without a normal distribution, respectively. Distribution of the variables was analyzed by construction of histograms and by the Shapiro-Wilk $W$ test. A p-value <0.05 was considered significant. Differences between each rNAPc2 dose group and the placebo group were tested for all study outcomes. Comparison of baseline and procedural characteristics, clinical events, and the incidences of minor and major bleedings was by $\chi^2$ analysis for categorical variables and either the unpaired Student’s $t$ test or Mann-Whitney $U$ test for continuous variables as appropriate. Regarding thrombin generation markers, the treatment groups were compared for each time point separately by means of an one-way analyses of variance (ANOVA) and subsequent pairwise comparisons with placebo, using Bonferroni correction. Spearman correlation was computed between FCT and rNAPc2 plasma levels at the moment of sheath removal, and between plasma levels of rNAPc2 and markers of thrombin generation after PCI. All statistical analyses were performed with SPSS 10.1 (Chicago, Illinois).

**Results**

**Patients**

A total of 154 patients were randomized to 1 of 4 rNAPc2 dosages or placebo. Baseline characteristics are shown in Table 1. No significant differences were found between each rNAPc2 treatment group and the placebo group, except for gender in the second 3.5 $\mu$g/kg dose group.
Table 1. Baseline characteristics

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Placebo (N=30)</th>
<th>3.5* (N=23)</th>
<th>3.5 (N=24)</th>
<th>5.0 (N=24)</th>
<th>7.5 (N=27)</th>
<th>10.0 (N=26)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (y): mean ± SD</td>
<td>59 ± 8</td>
<td>63 ± 9</td>
<td>58 ± 6</td>
<td>60 ± 13</td>
<td>61 ± 8</td>
<td>59 ± 9</td>
</tr>
<tr>
<td>Male gender</td>
<td>67%</td>
<td>87%</td>
<td>92%†</td>
<td>79%</td>
<td>70%</td>
<td>85%</td>
</tr>
<tr>
<td>Previous MI: N (%)</td>
<td>9 (30)</td>
<td>8 (35)</td>
<td>7 (29)</td>
<td>8 (33)</td>
<td>8 (30)</td>
<td>8 (31)</td>
</tr>
<tr>
<td>Previous PCI: N (%)</td>
<td>4 (13)</td>
<td>7 (30)</td>
<td>3 (12)</td>
<td>2 (8)</td>
<td>9 (33)</td>
<td>6 (23)</td>
</tr>
<tr>
<td>Previous CABG: N (%)</td>
<td>1 (3)</td>
<td>2 (8)</td>
<td>1 (4)</td>
<td>3 (17)</td>
<td>3 (11)</td>
<td>1 (4)</td>
</tr>
<tr>
<td>1-vessel disease: N (%)</td>
<td>17 (57)</td>
<td>10 (43)</td>
<td>14 (58)</td>
<td>11 (46)</td>
<td>18 (67)</td>
<td>15 (58)</td>
</tr>
<tr>
<td>2-vessel disease: N (%)</td>
<td>9 (30)</td>
<td>7 (30)</td>
<td>10 (42)</td>
<td>12 (50)</td>
<td>5 (19)</td>
<td>7 (27)</td>
</tr>
<tr>
<td>3-vessel disease: N (%)</td>
<td>4 (13)</td>
<td>6 (26)</td>
<td>0 (0)</td>
<td>1 (4)</td>
<td>4 (15)</td>
<td>4 (15)</td>
</tr>
<tr>
<td>Body Mass Index (kg/m²): mean ± SD</td>
<td>28 ± 4</td>
<td>27 ± 3</td>
<td>28 ± 4</td>
<td>28 ± 3</td>
<td>26 ± 3</td>
<td>26 ± 2</td>
</tr>
<tr>
<td>Hypertension: N (%)</td>
<td>9 (30)</td>
<td>3 (13)</td>
<td>6 (25)</td>
<td>7 (29)</td>
<td>5 (19)</td>
<td>7 (27)</td>
</tr>
<tr>
<td>Hypercholesterolemia: N (%)</td>
<td>13 (43)</td>
<td>10 (43)</td>
<td>9 (37)</td>
<td>10 (42)</td>
<td>10 (37)</td>
<td>9 (35)</td>
</tr>
<tr>
<td>Diabetes: N (%)</td>
<td>5 (17)</td>
<td>1 (4)</td>
<td>1 (4)</td>
<td>2 (8)</td>
<td>4 (15)</td>
<td>4 (15)</td>
</tr>
</tbody>
</table>

*No clopidogrel administration pre-PCI. †P value vs placebo <0.05. χ² analysis for categorical variables and Student’s t test for continuous variables. CABG=coronary artery bypass grafting; MI=myocardial infarction; PCI=percutaneous coronary intervention.

Procedural characteristics

Procedural characteristics are presented in Table 2. The mean time between study drug administration and access to the femoral artery was 3.4 ± 1.4 hours. Clopidogrel was administered in 74% of the study population. In 28% of these patients, clopidogrel was started prior to PCI, with no differences between placebo and each rNAPc2 dose group.

Femoral compression time was evaluated in 149 (97%) patients. Compression time was not measured in four patients and one patient was excluded because PCI and sheath removal was performed 24 hours after study drug administration. The introduction of the sheath in the femoral artery was uneventful in all patients except one that required multiple punctures. As shown in Figure 1, a trend toward an increasing FCT was shown in the two highest rNAPc2 treatment groups, with a statistically significant difference between the highest dose group (i.e. 10.0 μg/kg) and placebo (median=16 min (IQR: 10–28) and median=10 min (IQR: 10–14), respectively, p<0.001). Spearman correlation showed a significant correlation between FCT and rNAPc2 plasma levels at the moment of sheath removal (r=0.317, p<0.001).
Table 2. Procedural characteristics

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Placebo (N=30)</th>
<th>3.5* (N=23)</th>
<th>3.5 (N=24)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Duration PCI (min): median (IQR)†</td>
<td>14 (7-35)</td>
<td>22 (15-41);‡</td>
<td>14 (6-33)</td>
</tr>
<tr>
<td>Number of lesions per PCI</td>
<td>1.6</td>
<td>1.7</td>
<td>1.7</td>
</tr>
<tr>
<td>PCI site:</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>- RCA: N (%)</td>
<td>11 (37)</td>
<td>6 (26)</td>
<td>9 (37)</td>
</tr>
<tr>
<td>- LAD: N (%)</td>
<td>14 (47)</td>
<td>13 (57)</td>
<td>9 (37)</td>
</tr>
<tr>
<td>- LCx: N (%)</td>
<td>9 (30)</td>
<td>6 (26)</td>
<td>4 (17)</td>
</tr>
<tr>
<td>Patients with stent implantation(s): N (%)</td>
<td>21 (70)</td>
<td>16 (70)</td>
<td>12 (50)</td>
</tr>
<tr>
<td>Total dose of UFH (IU) during PCI: median (IQR)</td>
<td>5975 (5000-8560)</td>
<td>6000 (5500-7000)</td>
<td>6250 (5000-7000)</td>
</tr>
<tr>
<td>Administration of clopidogrel: N (%)</td>
<td>21 (70)</td>
<td>16 (70)</td>
<td>14 (58)</td>
</tr>
<tr>
<td>Clopidogrel administration prior PCI: N (%)</td>
<td>6 (20)</td>
<td>0 (0);‡</td>
<td>6 (25)</td>
</tr>
<tr>
<td>Guiding catheter size:</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>- 5 French: N (%)</td>
<td>4 (13)</td>
<td>6 (26)</td>
<td>5 (21)</td>
</tr>
<tr>
<td>- 6 French: N (%)</td>
<td>26 (87)</td>
<td>17 (74)</td>
<td>19 (79)</td>
</tr>
</tbody>
</table>

*No clopidogrel administration pre-PCI. †Period between first and last balloon inflation. ‡P value vs placebo <0.05, χ² analysis for categorical variables and Mann-Whitney U test for continuous variables. IQR=interquartile range; LAD=left anterior descending artery; LCx=left circumflex artery; PCI=percutaneous coronary intervention; RCA=right coronary artery; UFH=unfractionated heparin.

Figure 1. Femoral compression time (minutes, median ± interquartile range) after sheath removal from the femoral artery. *No clopidogrel administration pre-PCI. The interquartile ranges of the 3.5* and the 5.0 rNAPc2 dose groups are from 10 to 10 minutes. Comparison between placebo and each rNAPc2 dose group by Mann-Whitney U test. PCI=percutaneous coronary intervention.

Clinical events

Clinical event rates were low and not apparently related to the rNAPc2 dose. Intervention related MI’s were observed in 10 patients (6% of total population). Two
patients experienced an acute MI after hospital discharge. Bail-out stent implantation was performed in two patients, with four patients experiencing transient vessel closure during the intervention. Two patients underwent coronary bypass grafting (CABG), one of which was an emergency procedure after PCI. There were two deaths, one due to cardiogenic shock six days following the procedure and the second due to suspected cerebral vascular accident (CVA) that occurred one day following angioplasty. The clinical diagnosis of CVA was not confirmed by CT-scan or autopsy. The patient had a history of ischemic stroke. There were no arteriovenous fistulae or femoral aneurysms. Differences in event rates between each rNAPc2 treatment group and the placebo population were not statistically significant.

**Bleeding events**

As presented in Table 3, there was no difference between the first three rNAPc2 dosage levels and placebo with respect to minor bleeding episodes, varying from 1 to 3 bleedings per patient group. This incidence of minor bleeding increased significantly to seven events in the highest rNAPc2 dose group (p<0.01). The majority (n=4) of these seven bleeding episodes were late re-bleeds at the site of sheath removal occurring >2 hours after hemostasis.

There were four episodes of major bleedings, of which three occurred in patients treated with 5.0 μg/kg of rNAPc2 (Table 3). The three incidents were classified as excessive drainage after emergency CABG, sustained oral oozing after tracheal intubation, and suspected CVA. In addition to aspirin, UFH and clopidogrel, each of these three patients also received a GP IIb/IIIa antagonist. Two received abciximab and one patient was treated with tirofiban. It should be noted that two patients, one each in the 3.5 and 7.5 μg/kg dose groups, also received a GP IIb/IIIa antagonist (both treated with eptifibatide) and had an uneventful outcome. The fourth major hemorrhage was an esophageal bleeding in a patient in the 7.5 μg/kg dose group,
Table 3. Incidence of bleeding

<table>
<thead>
<tr>
<th>Bleeding, N (%)</th>
<th>Placebo (N=30)</th>
<th>3.5* (N=23)</th>
<th>3.5 (N=24)</th>
<th>5.0 (N=24)</th>
<th>7.5 (N=27)</th>
<th>10.0 (N=26)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Minor</td>
<td>2 (6.7)</td>
<td>2 (8.7)</td>
<td>1 (4.2)</td>
<td>1 (4.2)</td>
<td>3 (11.1)</td>
<td>7 (26.9)†</td>
</tr>
<tr>
<td>Major</td>
<td>0 (0.0)</td>
<td>0 (0.0)</td>
<td>0 (0.0)</td>
<td>3 (12.5)†</td>
<td>1 (3.7)</td>
<td>0 (0.0)</td>
</tr>
</tbody>
</table>

*No clopidogrel administration pre-PCI. †P value vs placebo <0.05, χ² analysis.
PCI=percutaneous coronary intervention.

suffering from esophagitis (confirmed by endoscopy). This patient received aspirin, UFH and clopidogrel but no GP IIb/IIIa antagonist.

**Thrombin generation**

Sixteen patients (10.4%) were excluded for the secondary outcome analysis because coronary angiography was not followed by angioplasty (n=6), PCI was performed 24 hours after study drug administration (n=1), diagnosis of unstable angina just prior to PCI (n=1), and either GP IIb/IIIa antagonist or UFH administration after the procedure (n=8). As shown in Figure 2, F₁₋₂ plasma levels were lower in all treatment groups during the procedure coincident with UFH administration. A gradual 1.4 fold increase above pre-procedural levels of F₁₋₂ was observed over the 30 hours observation period after the intervention in the placebo group only. There was continued suppression of thrombin generation in all rNAPc2 dose groups that differed significantly from the placebo group at 24 hours out to 36 hours following a single subcutaneous administration. The extended duration of the PCI in the first 3.5 µg/kg dose group did not affect these results.

The F₁₋₂ results were mirrored by the levels of TAT complexes although this was statistically significant only at the two highest doses at 36 hours, which was most probably due to the wide variation between the subjects (data not shown).

There was a dose-dependent increase in rNAPc2 plasma levels. A weak inverse correlation was shown after PCI, at 12 and at 24 hours between plasma levels of rNAPc2 and the thrombin generation markers F₁₋₂ and TAT complexes. However, this became statistically significant for both markers at 36 hours (r = -0.300 and r = -0.439, respectively, p<0.01). Figure 3 presents the inverse relation between rNAPc2 plasma levels and thrombin generation, as measured by F₁+2, at 36 hours.
Figure 2. Mean values (± SEM) of F$_{1+2}$ plasma levels for the placebo and rNAPc2 dose groups. *No clopidogrel treatment pre-PCI. The following statistical significant differences were found between rNAPc2 dose groups and placebo (Bonferroni corrected p-values, including 5 comparisons at each time point): *p<0.05: 3.5† vs 3.5 vs placebo, p<0.01: 7.5 vs placebo; **p<0.05: 5.0 vs placebo, p<0.01: 3.5† vs placebo, p<0.005: 3.5, 7.5, and 10.0 vs placebo; ***p<0.05: 3.5†, 3.5 and 5.0 vs placebo, p<0.001: 7.5, and 10.0 vs placebo. F$_{1+2}$=prothrombin activation fragment 1+2; PCI=percutaneous coronary intervention; rNAPc2=recombinant Nematode Anticoagulant Protein c2.

Figure 3. rNAPc2 plasma levels in relation to plasma levels of F$_{1+2}$ at 36 hours. F$_{1+2}$=prothrombin activation fragment 1+2; rNAPc2=recombinant Nematode Anticoagulant Protein c2.
Discussion
In patients with stable angina undergoing planned coronary stent implantation, the composite of death, MI, urgent target revascularisation and bailout GP IIb/IIIa inhibitor therapy has been reported to occur in approximately 7% of cases within 48 hours. The composite of death, MI and urgent target revascularisation increases to 9%-13% within 48 hours after PCI in patients with unstable coronary syndromes. Tissue factor/factor VIIa plays a pivotal role in initiating the thrombotic response to vascular injury, which makes this enzymatic complex a target for the development of anticoagulant strategies for patients suffering from coronary thrombosis. Our study demonstrates that inhibition of the TF/factor VIIa complex by a single subcutaneous dose of rNAPc2, administered prior to elective coronary angioplasty, is safe in combination with aspirin, clopidogrel and UFH and produces a prolonged and significant suppression of thrombin generation.

Bleeding events
Overall, rNAPc2 was well tolerated with respect to the incidence of major and minor bleeding episodes. However, of four major bleedings, three occurred in patients of the 5.0 μg/kg dose group at the time they also received a GP IIb/IIIa antagonist. Two additional patients also received GP IIb/IIIa antagonists and did not experience a bleeding. As the number of observations in patients receiving the combination of rNAPc2 with GP IIb/IIIa antagonists is small, no conclusion can be drawn regarding the safety of this combination. Therefore, additional safety data from future trials, in which rNAPc2 and GP IIb/IIIa antagonists are used concomitantly, will be required prior to defining how these strategies can be optimally used in the treatment of patients. The effects of rNAPc2 during coronary bypass surgery remain to be evaluated since only two study patients underwent this procedure after receiving rNAPc2. As expected, there was a higher incidence of minor bleeding episodes in the two highest dose groups, with a statistically significant difference between the 10.0 μg/kg and placebo group. The majority of the minor bleedings in the 10.0 μg/kg dose group were late re-bleeds at the catheter access site, consistent with the prolonged anticoagulant effect of rNAPc2. While recombinant factor VIIa has been shown to transiently reverse the anticoagulant effects of rNAPc2 in normal volunteers, there were no incidents of severe bleeding that required its use.

Femoral compression time
In patients undergoing cardiac catheterization, bleeding after sheath removal at the
arterial puncture site is a form of controlled surgical bleeding that may be quantified, similar to the conventional bleeding time according to Mielke. To this end, we standardized sheath removal and quantified the time required to complete hemostasis using a protocol driven procedure. In our study, the FCT was prolonged in the 7.5 and 10.0 μg/kg dose groups with a statistically significant difference between the latter and placebo. Although little interindividual variations may persist despite a structured protocol, these data still suggest a dose-dependent prolongation, and an apparent effect on clinical hemostasis at the two highest doses of rNAPc2. In addition, there was a significant correlation between FCT and rNAPc2 plasma levels at the time of sheath removal. Systemic F$_{1+2}$ and TAT complex plasma levels were maximally suppressed already at the lowest dose of rNAPc2, and there was no correlation between these plasma markers and FCT. Clearly, hemostasis involves contributions of the arterial wall, platelets and coagulation, and systemic coagulation marker plasma levels may not completely reflect this process. Thus, based on our findings, the FCT may provide clinical relevant quantitative information that is clinically relevant. Indeed, this has to be confirmed in future trials. However, this needs to be confirmed in future trials.

**Thrombin generation**

Although the patients enrolled in this study were not considered to be high-risk, in the placebo group we observed a marked and sustained elevation of thrombin generation, as measured by plasma levels F$_{1+2}$, to above pre-procedural levels up to at least 30 hours after PCI. In contrast, thrombin generation continued to be suppressed in all of the rNAPc2 treatment groups, which was sustained through the last sampling time point at 36 hours. These data suggest that the ongoing thrombin generation observed in the placebo group following the procedure was effectively suppressed by rNAPc2. Plasma levels TAT complexes, used as another measure of thrombin generation, showed as expected a similar response as found for F$_{1+2}$. Thus, TF/factor VIIa may play an important role in the process of ongoing thrombosis after PCI, as has been suggested recently in high risk patients with unstable coronary syndromes, and rNAPc2 appears to effectively suppress this process.

Unfractionated heparin and low-molecular weight heparins are widely used for the treatment of patients suffering from acute coronary syndromes or undergoing PCI. A synthetic pentasaccharide (fondaparinux), which may serve as an alternative for both drugs, is currently undergoing clinical development in these patient populations.
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However, it should be noted that although these agents should theoretically attenuate thrombin generation via the inhibition of factor Xa assembled in the prothrombinase complex (i.e. factor Va, negatively charged membrane surfaces and calcium ions), the dependence on antithrombin-III may limit their effectiveness in situations where there is a high level of prothrombinase activity, such as the site of an atherosclerotic plaque rupture, since it has been shown that antithrombin-III dependent inhibitors, including heparins of any molecular size, do not effectively inhibit factor Xa assembled in the prothrombinase complex\textsuperscript{29}. Therefore, this may explain the reduced antithrombotic potential of these agents in suppressing the thrombogenicity of coronary lesions leading to clinical events during or shortly after acute therapy\textsuperscript{21,22}. In contrast, the inhibition of TF/factor VIIa-mediated generation of factor Xa and subsequently thrombin by rNAPc2 is enhanced following the assembly of the TF/factor VIIa complex\textsuperscript{9}. Therefore, rNAPc2 has the potential advantage of attenuating the highly amplified formation of thrombin by inhibiting the coagulation cascade at a more proximal site relative to direct inhibitors of factor Xa and thrombin, and thus, may offer a more effective approach in reducing thrombosis in patients with coronary artery diseases.

**Optimal rNAPc2 dose**

As mentioned, the suppression of plasma levels of F\textsubscript{1,2} and TAT complexes by rNAPc2 was already maximal at the lowest dose (i.e. 3.5 \mu g/kg). The significant inverse correlations between plasma levels of rNAPc2 and each of these two markers at 36 hours indicates that the two highest doses of rNAPc2 results in a more sustained suppression of both markers compared to lower doses (see also Figures 2 and 3). However, the usefulness of the systemic markers of thrombin generation in assessing the clinical efficacy of rNAPc2 could not be determined in this study. The effect of rNAPc2 on the observed clinical bleeding complications suggests that the optimal and sufficiently safe dose of rNAPc2, when combined with aspirin, clopidogrel and unfractionated heparin, is likely to be below 10.0 \mu g/kg. This is consistent with the significant prolongation of the FCT at the highest dose.

**Conclusion**

Our study indicates that inhibition of the TF/factor VIIa complex at doses up to 7.5 \mu g/kg of rNAPc2 in combination with aspirin, clopidogrel and unfractionated heparin, appears to be a safe and effective strategy to prevent thrombin generation in patients undergoing catheter based coronary intervention. These results are supportive of
advancing rNAPc2 into a phase II dose-ranging trial in patients suffering from unstable angina or non-Q-wave myocardial infarction.

Appendix

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Sponsor: Corvas International, Inc: G. Vlasuk, PhD; W. Rote, PhD; J. Magpantay; and M. Stannard.
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