Anticoagulation in severe sepsis and the multiple organ dysfunction syndrome

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Chapter 2

Recombinant nematode anticoagulant protein c2, an inhibitor of tissue factor/factor VIIa, attenuates coagulation and the interleukin-10 response in human endotoxemia

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Chapter 2

Abstract

Background
The tissue factor-factor (F)VIIa complex (TF/FVIIa) is responsible for the initiation of blood coagulation under both physiological and pathological conditions. Recombinant Nematode Anticoagulant Protein c2 (rNAPc2) is a potent inhibitor of TF/FVIIa, mechanistically distinct from tissue factor pathway inhibitor. The first aim of this study was to elucidate the pharmacokinetics and pharmacodynamics of a single intravenous (i.v.) dose of rNAPc2. The second aim was to study its effect on endotoxin-induced coagulation and inflammation.

Methods and Results
In the first part of the study, rNAPc2 was administered to healthy volunteers in three different doses. There were no safety concerns and the pharmacokinetics were consistent with previous studies, in which rNAPc2 was administered subcutaneously. rNAPc2 elicited a dose-dependent reduction of the endogenous thrombin potential and a selective prolongation of prothrombin time. In the second part of the study, the effect on endotoxin-induced coagulation and inflammation was studied. The administration of rNAPc2 completely blocked the endotoxin-induced thrombin generation, as measured by plasma prothrombin fragment F1+2. The endotoxin-induced effect on fibrinolytic parameters such as plasmin-antiplasmin complexes and plasminogen activator inhibitor type 1 was not affected by rNAPc2. The administration of rNAPc2 attenuated the endotoxin-induced rise in interleukin(IL)-10, without affecting the rise in other cytokines.

Conclusions
rNAPc2 is a potent inhibitor of TF/FVIIa, which was well tolerated and could safely be used intravenously in this Phase I study in healthy male volunteers. A single i.v. dose rNAPc2 completely blocked endotoxin-induced thrombin generation without affecting the fibrinolytic response. In addition, rNAPc2 attenuated the endotoxin-induced rise in IL-10, without affecting the rises in other cytokines.
**Introduction**

Tissue factor (TF) is the major cellular trigger of blood coagulation under physiological conditions [1]. It has been well established that TF also plays an important role in disease processes resulting from the activation of the coagulation pathway. For example, since TF is present in atherosclerotic plaques, acute coronary syndromes are believed to be the consequence of pathological plaque rupture with subsequent thrombus formation initiated by the TF/factor (F)VIIa complex (TF/FVIIa) [2]. In addition, TF levels are elevated during sepsis and this is believed to contribute directly to the pathogenesis of multiple organ failure [1]. A variety of inflammatory stimuli, including bacterial cell products and cytokines, are known to induce the expression of TF on the surface of endothelial cells and monocytes, thereby activating the coagulation pathway [3]. In view of the central role of TF/FVIIa in the activation of coagulation, novel therapeutic modalities aimed at inhibiting this complex are currently being evaluated in experimental and clinical trials.

The major physiological inhibitor of TF/FVIIa is the Kunitz-type protease inhibitor, tissue factor pathway inhibitor (TFPI). TFPI binds to and inhibits factor (F)Xa directly, prior to forming a quaternary inhibitory complex with TF/FVIIa, thereby inhibiting thrombin generation. Experimental settings where animals were depleted of TFPI have a demonstrated sensitivity to TF and endotoxin and a propensity to develop intravascular coagulation [1]. In a lethal *Escherichia coli* sepsis model in baboons, treatment with TFPI attenuated the procoagulant and interleukin(IL)-6 response and prevented mortality [4]. However, in healthy human volunteers administered endotoxin, blocking TF/FVIIa with TFPI had no impact on inflammatory cytokines, but completely prevented endotoxin-induced activation of coagulation [5]. A phase II clinical trial of recombinant TFPI (rTFPI) in patients with severe sepsis demonstrated accelerated decrease of IL-6 plasma levels in the rTFPI group [6]. However, the recently published phase III trial of rTFPI in severe sepsis, OPTIMIST, failed to show a reduction in the primary endpoint of 28-day all-cause mortality [7].

Recombinant Nematode Anticoagulant Protein c2 (rNAPc2) is an 85 amino acid protein that was originally isolated from the hematophagous hookworm *Ancylostoma caninum* [8]. It specifically inhibits TF/FVIIa by a unique mechanism of action that requires the initial binding of rNAPc2 to the serine protease FXa or its circulating zymogen FX prior to formation of the quaternary inhibitory complex with TF/FVIIa [9,10]. The utilization of zymogen FX as an inhibitory scaffold obviates the need for generating activated FX to inhibit TF/FVIIa. In addition, the formation of a complex between rNAPc2 and FX results in an elimination half-life of >50 h in man [10]. In a model of endotoxin-induced coagulation activation in chimpanzees, rNAPc2 completely inhibited thrombin generation [11]. Clinically, rNAPc2 was shown to be effective in the prevention of
postoperative venous thromboembolism in patients undergoing total knee replacement [12]. In addition, TF/FVIIa inhibition with rNAPc2 was also shown to be a safe and potentially effective strategy to prevent thrombin generation in patients undergoing elective coronary interventions [13]. In all these studies, rNAPc2 was administered subcutaneously. The current study had two aims: (i) to study the pharmacokinetics of a single intravenous (i.v.) dose of rNAPc2, and (ii) to study the effect of a single i.v. dose of rNAPc2 on endotoxin-induced coagulation and inflammatory responses in healthy human volunteers.

**Materials and methods**

**Study design**
The study protocol was conducted according to the International Conference on Harmonisation Good Clinical Practice Guideline and was approved by the Institutional Review Board. Twenty-eight healthy men (age 18–35 years) volunteered to participate in the study. Written informed consent was obtained from each subject. None of the subjects had abnormalities on physical examination or routine laboratory investigation. The subjects did not take any medication and did not smoke or use illicit drugs. In the first part of the study, safety and pharmacokinetics (PK) of rNAPc2 were evaluated in three groups of four subjects. All subjects fasted overnight before administration of rNAPc2. Each group of subjects received rNAPc2 (Corvas Int., San Diego CA, USA) as a single i.v. dose of 3.0, 5.0 or 7.5 µg/kg, respectively. Following analysis of the safety and PK data, the dose of 7.5 µg/kg was chosen for the second part of the study, in which sixteen subjects participated. The dose of 7.5 µg/kg was chosen because this dose elicited the highest prothrombin time (PT) elevation (1.5 times baseline), which has proved to be safe in studies in which rNAPc2 was administered subcutaneously [14,15]. Eight subjects received endotoxin alone and eight subjects received the combination of endotoxin and rNAPc2. All subjects fasted overnight before endotoxin administration. The endotoxin used in both groups (*E. coli* lipopolysaccharide, lot G-1; US Pharmacopeia, Rockville, MD, USA) was administered as a single i.v. dose of 4 ng/kg bodyweight. The combined treatment group received rNAPc2 as a single i.v. dose of 7.5 µg/kg bodyweight, immediately followed by endotoxin. Oral temperature, blood pressure, heart rate and oxygen saturation were measured at hourly intervals for 24 h, and once daily through 72 h (Dinamap 1846 SX; Criticon, Tampa, FL, USA). Clinical symptoms such as headache, shivering, nausea, vomiting, tiredness and malaise were recorded at hourly intervals for the first 24 h, and once daily through 72 h, using a graded scale (0=absent, 1=weak, 2=moderate, 3=severe). Electrocardiograms were performed at baseline, 24 h, and 8 days after the start of the study. To detect adverse events, clinical follow-up consisting
of a complete history and physical examination was performed at 1, 2, 3 and 8 days after the start of the study.

**Blood collection**

Blood was collected from an i.v. cannula at 10 min before, at 5, 15 and 30 min and at 1, 1.5, 2, 3, 4, 6, 8, 12, 24, 48 and 72 h after endotoxin administration. Blood for cytokine assays and leukocyte counts was collected in K3-EDTA-containing tubes. All other blood samples were collected in citrated vacutainer tubes. Plasma was prepared by centrifugation of blood at 1800 g for 20 min at 16°C, followed by storage at -20°C until assays were performed.

**Laboratory tests**

The plasma concentration of rNAPc2 was analyzed by sandwich ELISA using an affinity-purified polyclonal antibody raised against rNAPc2. Detection of the captured rNAPc2 was by using the same antibody conjugated to horseradish peroxidase. Leukocyte counts and differentials were assessed by a Cell-dyn 4000 analyzer (Abbott Labs, Abbott Park, IL, USA). Inhibition of TF/FVIIa activity was measured on the Behring Coagulation System (BCS) according to a method for the determination of TFPI activity as described by Sandset et al. [16]. The endogenous thrombin potential (i.e. the time integral of thrombin generated in plasma in which coagulation is initiated via TF/FVIIa) was determined as described by Rosing et al. [17]. Coagulation assays [PT and activated partial thromboplastin time (APTT)] were performed on an automated coagulation analyzer (BCS) with reagents and protocols from the manufacturer (Dade Behring, Marburg, Germany). The plasma concentrations of prothrombin fragment F1+2 and plasmin-antiplasmin complexes (PAP) were measured by ELISA (Dade Behring). Plasminogen activator inhibitor type 1 (PAI-1) antigen was assayed by ELISA (Innotest PAI-1; Hyphen BioMed, Andrésy, France). Tumor necrosis factor-alpha (TNF-α), IL-6, IL-8 and IL-10 were measured using commercial ELISA kits (Central Laboratory of the Netherlands Red Cross Blood Transfusion Service, Amsterdam, the Netherlands).

**Pharmacokinetic analysis**

Pharmacokinetic analysis was performed assuming a non-compartmental model using WinNonlin 3.2 software (Carey, NC, USA). The linear up/log down trapezoidal method with interpolation was employed. Lambda z was estimated by the default curve stripping approach in WinNonlin. The mean ± SD were estimated using WinNonlin.

**Statistical analysis**

Data were analyzed using the Statistical Package for the Social Sciences (SPSS) for Windows, version 11.0 (SPSS, Chicago, IL, USA). Differences between the rNAPc2 and
control experiments were tested by analysis of repeated measures, using mixed linear models. Changes in time within the same group were analyzed by one-way analysis of variance. Values are given as means ± SEM. Significance was defined as \( p < 0.05 \).

**Results**

**Safety and pharmacokinetics of rNAPc2**

There were no safety or tolerability concerns following the i.v. dosing of rNAPc2 over the dose range from 3.0 - 7.5 \( \mu \)g/kg. No adverse events attributable to rNAPc2 infusion were observed, and no episodes of increased bleeding occurred. The PK elimination profile was consistent with previous studies in which rNAPc2 was administered subcutaneously (see Table 1).

**Table 1. Summary of pharmacokinetic parameters for rNAPc2**

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Dose 1 (3.0 ( \mu )g/kg)</th>
<th>Dose 2 (5.0 ( \mu )g/kg)</th>
<th>Dose 3 (7.5 ( \mu )g/kg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>( T_{\text{max}} ) (h)</td>
<td>1.4 ± 1.8</td>
<td>2.3 ± 3.8</td>
<td>0.6 ± 0.9</td>
</tr>
<tr>
<td>( C_{\text{max}} ) (( \mu )g/l)</td>
<td>47.2 ± 3.4</td>
<td>72.3 ± 6.6</td>
<td>136.7 ± 30.1</td>
</tr>
<tr>
<td>Half-life (h)</td>
<td>56.4 ± 6.4</td>
<td>55.8 ± 5.1</td>
<td>51.3 ± 6.7</td>
</tr>
<tr>
<td>( V_d ) (ml)</td>
<td>72.0 ± 4.9</td>
<td>76.3 ± 8.3</td>
<td>75.7 ± 18.7</td>
</tr>
<tr>
<td>( C_{\text{obs}} ) (ml/h/kg)</td>
<td>0.89 ± 0.10</td>
<td>0.96 ± 0.18</td>
<td>1.03 ± 0.18</td>
</tr>
<tr>
<td>LambdaZ (/h)</td>
<td>0.012 ± 0.001</td>
<td>0.013 ± 0.001</td>
<td>0.014 ± 0.003</td>
</tr>
<tr>
<td>( AUC_{\text{inf}} ) (( \mu )g h/l)</td>
<td>3391 ± 324</td>
<td>5367 ± 1119</td>
<td>7471 ± 1283</td>
</tr>
<tr>
<td>( T_{\text{last}} ) (h)</td>
<td>72</td>
<td>72</td>
<td>72</td>
</tr>
<tr>
<td>( C_{\text{last}} ) (( \mu )g/l)</td>
<td>17.4 ± 1.3</td>
<td>27.1 ± 5.4</td>
<td>37.2 ± 7.2</td>
</tr>
</tbody>
</table>

rNAPc2, recombinant Nematode Anticoagulant Protein c2; i.v., intravenous; \( T_{\text{max}} \), Time of maximum concentration; \( C_{\text{max}} \), maximum concentration; \( V_d \), apparent volume of distribution; \( C_{\text{obs}} \), clearance estimated from the observed concentrations; LambdaZ, terminal elimination rate constant; \( AUC_{\text{inf}} \), total area under the concentration-time curve to infinite time; \( T_{\text{last}} \), time of the last measured concentration; \( C_{\text{last}} \), last measured concentration. All data are expressed as mean ± SD.

**Clinical features and neutrophil response**

Injection of endotoxin induced a transient febrile response, together with a tachycardia and flu-like symptoms including headache, nausea, malaise and chills. In addition, endotoxin administration elicited activation of neutrophilic granulocytes. None of these changes was affected by rNAPc2.
Inhibition of TF/FVIIa activity
Following the administration of rNAPc2 alone, a dose-dependent inhibition of TF/FVIIa activity was observed. The extent of TF/FVIIa inhibition did not increase after endotoxin administration. In contrast, TF/FVIIa inhibition rose significantly after 5 min, followed by a gradual decline up to 72 h following the administration of endotoxin and rNAPc2.

Endogenous thrombin potential
To evaluate the residual ability of thrombin generation in plasma after the administration of rNAPc2, we measured the endogenous thrombin potential (ETP). As shown in Figure 1, administration of rNAPc2 alone resulted in a significant, dose-dependent reduction of ETP, which was also observed following the administration of endotoxin and rNAPc2.

![Graph showing ETP after administration of endotoxin and rNAPc2.](image)

Figure 1. ETP after administration of endotoxin and rNAPc2. Left panel, effect on ETP of administration of a single i.v. dose rNAPc2 of 3 (□), 5 (●) or 7.5 (▼) μg/kg to healthy male volunteers. Right panel, effect on ETP of administration of a single i.v. dose of 4 ng/kg endotoxin (○) or the combination of a single i.v. dose of 4 ng/kg endotoxin (○) and a single i.v. dose of 7.5 μg/kg rNAPc2 (▲) to healthy male volunteers. Data represent mean ± SEM. ETP, endogenous thrombin potential; rNAPc2, recombinant Nematode Anticoagulant Protein c2.

Clotting times
As shown in Figure 2, the administration of rNAPc2 alone resulted in a significant, dose-dependent prolongation of the PT, which was also observed following the administration of endotoxin and rNAPc2, followed by a gradual decline to baseline after 24 h.
Administration of endotoxin resulted in a transient, 22% reduction in APTT. Administration of endotoxin and rNAPc2 resulted in a 12% prolongation of the APTT, followed by a decline to 83% baseline.

![Graph showing PT after administration of endotoxin and rNAPc2](image)

**Figure 2.** PT after administration of endotoxin and rNAPc2. Left panel, effect on PT of administration of a single i.v. dose of 3 (□), 5 (●) or 7.5 (▲) μg/kg rNAPc2 to healthy male volunteers. Right panel, effect on PT of administration of a single i.v. dose of 4 ng/kg endotoxin (○) or the combination of a single i.v. dose of 4 ng/kg endotoxin and a single i.v. dose of 7.5 μg/kg rNAPc2 (▲) to healthy male volunteers. Data represent mean ± SEM. PT, prothrombin time; rNAPc2, recombinant Nematode Anticoagulant Protein c2.

**Thrombin generation**
As shown in Figure 3, the administration of endotoxin resulted in an enhanced thrombin generation, as reflected by a significant increase in the levels of prothrombin activation fragment F1+2. The administration of rNAPc2 completely attenuated the endotoxin-induced rise in F1+2 to baseline levels.

**Activation of the fibrinolytic system**
Administration of endotoxin resulted in activation of the fibrinolytic system, as confirmed by a transient increase in plasma concentrations of PAP, followed by a transient increase in PAI-1. The administration of rNAP-c2 did not affect the endotoxin-induced release of PAP or PAI-1.
Figure 3. F1+2 after administration of endotoxin and rNAPc2. Effect on F1+2 of administration of a single i.v. dose of 4 ng/kg endotoxin (○) or the combination of a single i.v. dose of 4 ng/kg endotoxin and a single i.v. dose of 7.5 μg/kg rNAPc2 (▲) to healthy male volunteers. Data represent mean ± SEM. F1+2, prothrombin fragment F1+2; rNAPc2, recombinant Nematode Anticoagulant Protein c2.

Figure 4. IL-10 after administration of endotoxin and rNAPc2. Effect on IL-10 of administration of a single i.v. dose of 4 ng/kg endotoxin (○) or the combination of a single i.v. dose of 4 ng/kg endotoxin and a single i.v. dose of 7.5 μg/kg rNAPc2 (▲) to healthy male volunteers. Data represent mean ± SEM. IL-10, interleukin-10; rNAPc2, recombinant Nematode Anticoagulant Protein c2.
Cytokines
All measured cytokines (TNF-α, IL-6, IL-8 and IL-10) rose in response to endotoxin. TNF-α rose from below the detection limit (1.4 pg/ml) to 1706 ± 356 pg/ml 90 min after endotoxin administration. IL-6 rose from below the detection limit (0.6 pg/ml) to 4345 ± 991 pg/ml 3 h after endotoxin administration. IL-8 rose from below the detection limit (1 pg/ml) to 2562 ± 235 pg/ml 2 h after endotoxin administration. The administration of rNAPc2 did not affect the rise in TNF-α, IL-6 or IL-8.
As shown in Figure 4, IL-10 was significantly elevated, peaking at 3 h following endotoxin administration. The administration of rNAPc2 with endotoxin resulted in an attenuated IL-10 response, which reached statistical significance 3 h following the challenge.

Discussion
In the present clinical study, we examined the PK and pharmacodynamics (PD) of a single i.v. administration of rNAPc2, as well as its effect on endotoxin-induced coagulation and inflammatory responses in healthy human volunteers. We demonstrated that the PK and PD of intravenously and subcutaneously administered single doses of rNAPc2 are comparable [14,15]. The administration of rNAPc2 prevented endotoxin-induced thrombin generation, as reflected by the complete attenuation of the increase of F1+2. In addition, rNAPc2 attenuated the endotoxin-induced IL-10 response, without affecting the endotoxin-induced rise in TNF-α, IL-6 and IL-8.
The administration of rNAPc2 was well tolerated and there were no safety concerns. A single i.v. dose of rNAPc2 resulted in a dose-dependent TF/FVIIa inhibition and elicited a dose-dependent prolongation of PT, with a maximum elevation of approximately 1.5-fold over the baseline value at a dose of 7.5 μg/kg. This dose-dependent, selective prolongation of the PT is consistent with the results of Vlasuk et al., who found a maximum PT elevation of approximately 1.8-fold over baseline values after a single subcutaneous dose of 5 μg/kg rNAPc2 [14].
The present results demonstrate that a single i.v. rNAPc2 dose of 7.5 μg/kg prevents endotoxin-induced thrombin generation, as reflected by the complete attenuation of the increase of F1+2. This finding is in accordance with two earlier studies showing that inhibition of TF/VIIa by TFPI [18] and active site-inhibited FVIIa (FVIIai) [19] dose dependently inhibited the endotoxin-induced thrombin generation in healthy humans. The ability of rNAPc2 to prevent endotoxin-induced thrombin generation has previously been shown in chimpanzees [11].
In accordance with earlier studies [11, 19], the endotoxin-induced thrombin generation was preceded by a short and rapid activation of the fibrinolytic system, as reflected by a rise in PAP levels, followed by a rise in PAI-1 levels. rNAPc2 did not affect this endotoxin-
induced activation of the fibrinolytic system. This finding confirms that during low-grade endotoxemia in humans, the fibrinolytic response occurs independently of thrombin generation.

As previously demonstrated for TFPI [18] and FVIIai [19], rNAPc2 did not influence the rise in TNF-α, IL-6 and IL-8 induced by the administration of low-dose endotoxin in these healthy human volunteers. TF/FVIIa has been shown to have direct pro-inflammatory effects independent of the activation of coagulation in man [20]. However, as evidenced by the rises in TNF-α, IL-6 and IL-8, antagonizing the catalytic activity of this complex with three inhibitors with differing mechanisms of action did not attenuate the pro-inflammatory response. This finding differs from the effects of anti-TF/FVIIa strategies in non-human primate models of sepsis. In baboons administered a lethal i.v. E. coli infusion, inhibition of TF/FVIIa with TFPI [4] or FVIIai [21], attenuated the IL-6 and lethal responses. This difference in efficacy of anti-TF/FVIIa strategies could be explained by the significant differences between the animal model and the clinical setting described here. For example, the human endotoxemia model can be considered a relatively mild stimulus using low-dose, purified bacterial endotoxin. This is reflected in the difference in maximum levels of IL-6, which are approximately 300 times higher in baboons administered an LD100 dose of E. coli compared with human volunteers administered endotoxin (1256 versus 4.3 ng/ml) [22]. Furthermore, low-dose endotoxin administration does not elicit a rise in endogenous TFPI, whereas TFPI is elevated in clinical sepsis [22].

Compared with other well acknowledged proinflammatory cytokines such as TNF-α and IL-6, rNAPc2 did attenuate the endotoxin-induced IL-10 response. To our knowledge, this is the first time IL-10 attenuation by inhibition of TF/FVIIa has been demonstrated in vivo. The interplay of TF/FVIIa and IL-10 is largely unknown. Although IL-10 is able to reduce the TF-activity in vitro [23], no data are available on the effect of TF/FVIIa on the IL-10 response during infection. Generally, IL-10 is considered a potent anti-inflammatory cytokine. Anti-inflammatory cytokines such as IL-10 impair local antibacterial effector mechanisms [24]. This explains why treatment with the IL-10 inhibitor AS101 significantly increased survival in septic mice after cecal ligation and puncture [25]. Moreover, IL-10 is a major denominator of the immunosuppressive effect in septic plasma. It has been postulated that persistently high plasma levels of IL-10 reflect a state of immunoparalysis, caused by deactivation of monocytes [24]. Several authors have described significantly higher IL-10 plasma levels in non-survivors of febrile illnesses compared with survivors [26,27]. In this respect, the attenuation of the IL-10 response by rNAPc2 might reflect a protective effect against immune suppression during endotoxemia. It is conceivable that the attenuation of the IL-10 response by rNAPc2 might play a role in human sepsis. Further studies are necessary to investigate this effect.
In conclusion, this study establishes the safety, tolerability and biological activity of i.v. rNAPc2 in healthy human volunteers. A single i.v. dose rNAPc2 of 7.5 μg/kg, completely blocks endotoxin-induced thrombin generation without affecting the fibrinolytic response. In addition, i.v. rNAPc2 attenuates the endotoxin-induced IL-10 response. The role of IL-10 suppression by rNAPc2 needs further investigation in human endotoxemia and sepsis.

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