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rNAPc2

Anticoagulant
TF-FVIIa Complex Inhibitor

rAcaNAPc2
Nematode anticoagulant protein c2

Recombinant protein NAP (nematode-extracted anticoagulant protein) (*Ancylostoma caninum* gene NAPc2 fragment)

EN: 236398

Abstract

Nematode anticoagulant protein c2 (rNAPc2) is a recombinant version of a naturally occurring protein that blocks the coagulation cascade by inhibiting the TF-FVIIa complex. Results from phase I clinical trials indicate that rNAPc2 is well tolerated following single and repeated s.c. and i.v. administration in healthy volunteers. No adverse events and no substantial bleeding episodes were observed. Clinical studies in patients showed that rNAPc2 is a safe and effective strategy for the prevention of thrombotic events after total knee replacement and elective coronary angioplasty. rNAPc2 is currently undergoing phase II investigation for the treatment of acute coronary syndrome. Moreover, the efficacy of rNAPc2 as a promising therapeutic option to inhibit coagulation activation in sepsis has been demonstrated in both animal models and patients. rNAPc2 has been also shown to protect against Ebola virus infection in primates, as well as to possess potential as an inhibitor of tumor growth *in vivo*.

Preparation

Nematode anticoagulant protein c2 (rNAPc2) is a small protein isolated from the parasitic blood-feeding hookworm *Ancylostoma caninum* and also produced by recombinant techniques using the yeast *Pichia pastoris*.

Background

Coronary artery disease, also called coronary heart disease, is the leading cause of death in developed countries and a major cause of emergency medical care and hospitalization. Coronary artery disease is caused by reduced blood supply to a portion of the myocardium, most often as a consequence of atherosclerotic plaque that narrows coronary artery lumen. Nondeveloped plaque allows sufficient flow of oxygen-rich blood to meet myocardial needs, but when plaque grows to a point where the blood supply cannot match the heart's demand, ischemia occurs. Over time, the thick plaque may rupture, exposing a thrombogenic surface upon which a platelet and fibrin thrombus is formed, worsening blood flow even more. If the thrombus totally blocks the blood supply to the heart muscle, it causes what is called an acute coronary syndrome (ACS), a wide-spectrum term used to cover a series of clinical conditions ranging from unstable angina to myocardial infarction (1).

Activation of the coagulation cascade and platelet aggregation are key events in the development of thrombi associated with ACS (1). The first event initiating blood coagulation after tissue injury is the exposure of blood to tissue factor (TF), a transmembrane protein that binds activated factor VII (FVIIa), which is present in trace amounts in circulating blood. This complex catalyzes the activation of factor IX (FIX) and factor X (FX). Factor IXa (FIXa) then binds to activated factor VIII (FVIIIa) to form a complex that activates FX. The principal role of FXa after being activated by TF-VIIa is to generate small amounts of thrombin in the proximity of platelets, enhancing their activation. Moreover, FXa promotes coagulation by binding to activated factor V (FVa) on membrane surfaces to form the prothrombinase complex. This complex first converts prothrombin (factor II; FII) to thrombin (factor IIa; FIIa), which converts fibrinogen to fibrin. Fibrin then polymerizes, forming the strand network of the developing clot (2-4). A model of these interactions is summarized in Figure 1.

Antiplatelet agents such as aspirin, clopidogrel and other nonsteroidal antiinflammatory drugs are used routinely in the prevention of ACS (5), but their inability to prevent thrombin generation may contribute to the remaining high rates of recurrent ischemic events after intense antithrombotic treatment in the acute phase. Anticoagulant therapies such as heparins, coumarin derivatives, thrombin or FXa inhibitors are effective for the prevention and treatment of thromboembolic diseases (6). However, the use of these agents is generally associated with side effects such as bleeding and thrombocytopenia (6, 7). Combination treatment with antiplatelet

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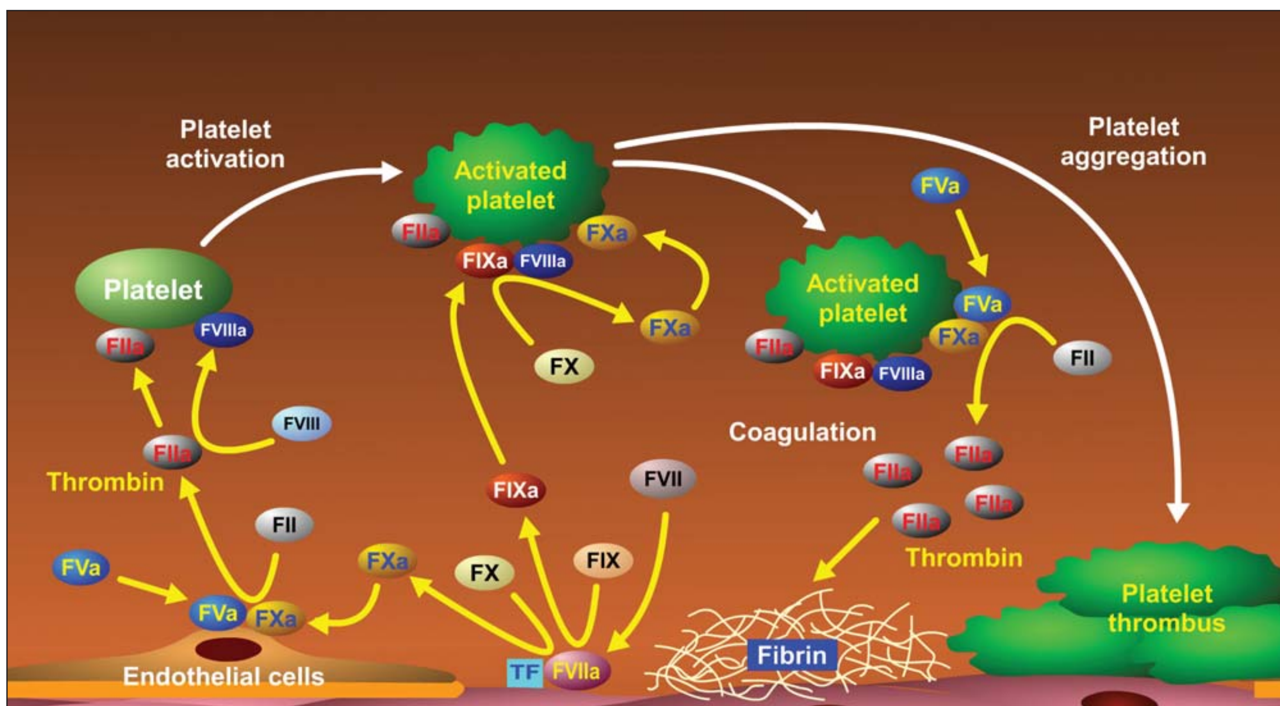

Fig. 1. The coagulation cascade and formation of a thrombus. After damage of vessel endothelium, contact occurs between blood and tissue factor (TF) exposed. TF binds activated plasma factor VII (FVIIa), forming the complex TF-FVIIa, which in turn binds and activates factors IX (FIXa) and X (FXa). This leads to the activation of small amounts of prothrombin (FII) to thrombin (FIIa), which locally activates platelets. Thrombin activates factor VIII (FVIIIa) which in turn directly activates FX on the surface of locally activated platelets. Activated factor X (FXa) promotes coagulation by binding to factor Va on the platelet membrane surface to form the prothrombinase complex. This complex converts large amounts of prothrombin to thrombin, which converts fibrinogen to fibrin. Activated platelets adhere to and aggregate on the damaged vessel wall. Together with the polymerized fibrin strand, aggregated platelets form a stable thrombus.

agents and anticoagulants provides improved efficacy in the secondary prevention of ACS (8), but the described limitations of these therapeutic approaches persist.

The search for new therapeutic alternatives has led to the development of rNAPc2 (recombinant nematode anticoagulant protein c2), a recombinant version of a naturally occurring protein, originally isolated form the hookworm *Ancylostoma caninum*, that has anticoagulant properties. Unlike heparin and its various derivatives which exert their effects at multiple later stages of the blood coagulation cascade, the anticoagulant effect of rNAPc2 results from its ability to block the TF-FVIIa protease complex (9, 10), potently inhibiting coagulation at its very starting point. rNAPc2 is currently undergoing phase II investigation for the treatment of ACS (11, 12). Additional potential indications for rNAPc2 include thoracic and vascular surgery, Ebola infection and cancer.

Preclinical Pharmacology

rNAPc2 binds initially with high affinity to a noncatalytic site on both FX (K_d = 0.87 nM) and FXa (K_d = 0.78 nM), using factor X as a scaffold for inhibition of the TF-FVIIa complex and finally forming a quaternary inhibitory complex (see Fig. 2). The docking of the FXa-rNAPc2 complex with TF-FVIIa is highly dependent on specific macromolecular interactions mediated by the γ-carboxyglutamic domain of FXa and the anionic phosphatidylserine head groups of the cell membrane. The canonical reactive loop of rNAPc2 docks into the active site of TF-FVIIa, forming the tightly bound quaternary complex TF-FVIIa-FXa-rNAPc2. Fluorescence binding studies show that natural NAPc2 does not interfere to a significant exent with the assembly of human prothrombinase complex (9, 10, 13-15).

The anticoagulant activity of rNAPc2 results in prolongation of the prothrombin time (PT) in human plasma at concentrations (< 60 nM) that do not significantly prolong the activated partial thromboplastin time (aPTT). At higher concentrations of rNAPc2 there is a prolongation of thromboplastin time resulting from the binding of the inhibitor to FXa (10, 16).

In addition to its activity as an antithrombotic agent for the prevention and treatment of acute thrombotic disorders, rNAPc2 may have other potential indications based on other roles of the TF-FVIIa complex beyond coagulation, including sepsis-induced coagulation, Ebola infection, angiogenesis and cancer.

The potential of rNAPc2 as a therapeutic option to inhibit coagulation activation in patients with sepsis was demonstrated in a model of endotoxin-induced coagulation activation in chimpanzees. Administration of a low

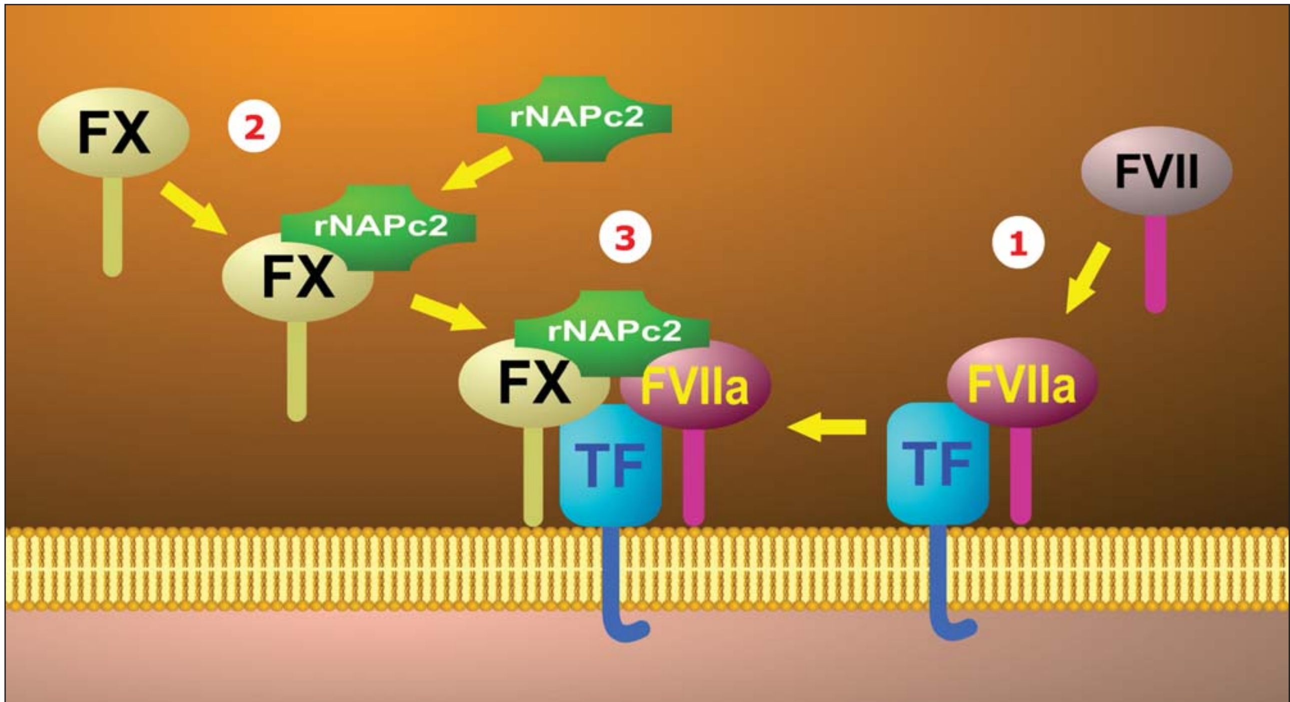

Fig. 2. Schematic sequential picture of the inhibitory mechanism of rNAPc2. First, coagulation is initiated when exposed tissue factor (TF) binds activated plasma factor VII (FVIIa), forming the complex TF-FVIIa (step 1). rNAPc2 then binds with high affinity to a noncatalytic site on both zymogen factor X (FX), or activated FX (step 2). Finally, this bimolecular complex forms a quaternary inhibitory complex with TF-FVIIa (step 3).

dose of endotoxin from *Escherichia coli* induced a marked increase in thrombin generation, as measured by plasma levels of prothrombin activation fragment F_{1+2} and thrombin-antithrombin complexes, which was completely blocked by rNAPc2 (10 mg/kg by s.c. bolus). In chimpanzees receiving rNAPc2 alone, there was a significant reduction in the activation of FX, but not of FIX, compared to animals receiving placebo. In contrast to the effect of rNAPc2 on thrombin generation, there was no effect of this inhibitor on the enhanced systemic fibrinolytic response induced by endotoxin (17).

A study to determine the role of TF-FVIIa complex inhibition by rNAPc2 in the host response to abdominal sepsis was performed in mice. Animals received an i.p. injection of live *E. coli* (10^4 cfu in 200 μl saline) with or without concurrent treatment with rNAPc2 (10 mg/kg s.c.). Results indicated that rNAPc2 strongly inhibited the activation of coagulation induced by peritonitis, measured as the levels of thrombin-antithrombin complexes and fibrin deposition in the liver and lungs. However, rNAPc2 did not alter bacterial outgrowth locally or dissemination of infection, and survival was not different between rNAPc2-treated and control mice. The inflammatory response, measured as histopathology, leukocyte recruitment to the peritoneal cavity and cytokine and chemokine levels, was not influenced by rNAPc2 treatment (18).

A rhesus monkey model was used to evaluate the effect of rNAPc2 on Ebola hemorrhagic fever infection. Results proved that postexposure protection with rNAPc2 against Ebola virus in primates provides a new foundation for therapeutic regimens that target the disease process rather than viral replication. rNAPc2 was administered to 9 monkeys either 10 min (n=6) or 24 h (n=3) after a high-dose lethal i.m. injection of Ebola virus Zaire 95 isolate (0.5 ml containing 1000 pfu). Both treatment regimens prolonged survival time, with a 33% survival rate in each group; mortality in the control group was near 100%. A significant increase in survival was observed for the 6 rNAPc2-treated monkeys that died (11.7 days compared with 8.3 days for untreated controls). rNAPc2 attenuated the coagulation response, as evidenced by modulation of various important coagulation factors, including plasma D dimers. Less prominent fibrin deposits and intravascular thromboemboli were observed in tissues of some animals that succumbed to the infection. Furthermore, rNAPc2 attenuated the proinflammatory response, with lower plasma concentrations of IL-6 and monocyte chemoattractant protein-1 (MCP-1) (19).

rNAPc2 has also been shown to possess significant potential as an antitumor agent. A study was aimed at assessing the role of coagulation inhibition by rNAPc2 on tumor growth *in vivo*. Mice were inoculated with tumor cells (2.5 x 10^4 Lewis lung carcinoma or B16 melanoma cells) via the tail vein and treated with increasing doses of rNAPc2. Results showed that rNAPc2 inhibited primary tumor growth in a dose-dependent manner, with an ED_{50} of 0.75 mg/kg/day i.p in the Lewis lung carcinoma model. rNAPc2 also exhibited potent inhibition of metastatic

tumor growth in the Lewis lung carcinoma model (T/C = 0.32 at 0.04-1 mg/kg) and the B16 melanoma model (T/C = 0.48 at 1 mg/kg i.p.). Moreover, rNAPc2 was a potent inhibitor of angiogenesis in the range of 40-160 μg/kg in the Matrigel plug assay. The data suggest that the proteolytic activity of TF-FVIIa promotes tumor growth and angiogenesis through a novel proangiogenic mechanism and independent of hemostasis. It should be noted that the antitumor activity of rNAPc2 was observed at doses significantly higher than those necessary for anticoagulant activity in clinical trials (20, 21).

Pharmacokinetics and Metabolism

In a double-blind, randomized, placebo-controlled study, 5 groups of 5-6 healthy male subjects received single doses of rNAPc2 ranging from 0.3 to 5 μg/kg s.c. or placebo (22), and in another study of similar design, 3 groups of 6 male and female subjects received repeated doses of rNAPc2 of 1.5, 3.5 or 5.0 μg/kg s.c. or placebo (23). Detectable levels of rNAPc2 in both studies were seen within 2 h after the first administration and slowly reached maximum concentrations (t_{max}) at 7-8.5 h following a single dose on day 1 and at 7-12 h following the last administration on day 5 after repeated administration. The mean elimination half-life ($t_{1/2\beta}$) of rNAPc2 was > 50 h and correlated directly with the prolongation of PT. The increases in mean C_{max}, AUC_{0-48h} or the $AUC_{0-\infty}$ values following single doses of 0.7-5.0 μg/kg or repeated doses of 1.5-5.0 μg/kg were proportional to dose. The mean C_{max} and AUC_{0-48h} values following the third dose on day 5 were markedly higher than those observed on day 1 at all 3 dose levels. The *in vivo* fate of rNAPc2, as defined by the elimination half-life, was principally regulated by its high-affinity interaction and binding to circulating FX. The stoichiometric binding of rNAPc2 to FX suggests hepatic mechanisms as the major route of clearance of the drug (22-25).

Clinical Studies

In phase I studies, a dose-dependent elevation of the PT was observed, reaching almost 4-fold above the baseline value after repeated doses of 5.0 μg/kg, and was directly correlated with rNAPc2 plasma concentrations. Activated partial thromboplastin time, thrombin time or template bleeding time showed little or no change and rNAPc2 was well tolerated (22-24).

An open-label study evaluated the safety, pharmacokinetics and pharmacodynamics of rNAPc2 in 3 groups of 4 healthy young men adminstered a single i.v. dose of 3.0, 5.0 or 7.5 μg/kg. Results showed no safety or tolerability concerns, and data were consistent with previous studies in which rNAPc2 was administered s.c. No adverse events or bleeding episodes were observed. Dose-dependent decreases in TF-FVIIa activation and prolongation of the PT were observed. Sixteen subjects were then administered endotoxin alone or with rNAPc2 (7.5 μg/kg i.v.). rNAPc2 completely blocked endotoxin-induced thrombin generation and attenuated the increase in IL-10, but had no effect on the fibrinolytic response to endotoxin (26).

Another open-label, multicenter phase II study was conducted to determine an effective and safe dose of rNAPc2 for the prevention of venous thromboembolism after elective unilateral total knee replacement in a total of 293 patients; 86% of patients were available for primary efficacy analysis. Five doses of rNAPc2 were tested (1.5, 3.0, 5.0, 1.5 and 3.0 μg/kg) as s.c. injections on the day of surgery and on days 3, 5 and 7. Primary efficacy outcome was a composite of overall deep vein thrombosis (DVT) and confirmed symptomatic venous thromboembolism recorded up to 48 h after the last dose of rNAPc2. The primary safety outcome was major bleeding up to 72 h after the last dose. A dose of 3.0 μg/kg administered within 1 h after surgery provided the best results, with an overall DVT rate of 12.2%, a proximal DVT rate of 1.3% and a major bleeding rate of 2.3% (27).

A multicenter, randomized, double-blind, placebo-controlled trial investigated the safety and pharmacodynamics of escalating doses of rNAPc2 in combination with aspirin, clopidogrel and unfractionated heparin in 154 patients undergoing elective coronary angioplasty. Results proved this strategy to be safe and effective for preventing thrombin generation. Patients received a single s.c. dose of rNAPc2 of 3.5, 5.0, 7.5 or 10.0 μg/kg 2-6 h before angioplasty. Results showed minor bleeding rates for the doses of 3.5-7.5 μg/kg comparable to those with placebo (6.7%), whereas a significant incidence of 26.9% was observed at the highest dose. Major bleeding occurred at 5.0 and 7.5 μg/kg in 3 and 1 patient, respectively. However, the group treated with 5.0 μg/kg also received a gpIIb/IIIa receptor antagonist at the moment of major bleeding. Systemic thrombin generation, as measured by prothrombin fragment 1+2 (F_{1+2}), was suppressed in all rNAPc2 dose groups to levels below pretreatment values for at least 36 h (28-30).

The ANTHEM (Anticoagulation with NAPc2 To Help Eliminate MACE)/TIMI 32 was a multicenter, double-blind, placebo-controlled phase IIa clinical trial of rNAPc2 in combination with other anticoagulants. In this study, rNAPc2 was evaluated in 200 patients with ACS, including unstable angina and non-S-T segment elevation myocardial infarction. Each patient was treated with 1 of 7 escalating i.v. doses of rNAPc2 (1.5, 2.0, 3.0, 4.0, 5.0, 7.5 and 10 μg/kg). The primary focus was to identify a safe and active dose as measured by major or minor hemorrhage occurring in the period from randomization to 7 days after the last dose of study drug, and the presence of ischemia. In the dose-confirmation phase, 25 patients received 10 μg/kg, the highest safe and active dose evaluated. All patients received low-molecular-weight heparin or unfractionated heparin and aspirin or clopidogrel. The study was completed in May 2005 and results showed that the addition of rNAPc2 to standard antithrombotic therapy led to a dose-related inhibition of thrombin generation without an increase in clinically significant bleeding. TIMI major or minor bleeding rate, the primary end-

point, showed no statistically significant difference between patients treated with rNAPc2 (4.3%) and those treated with placebo (2.5%). Prothrombin fragment 1+2, a marker of *de novo* thrombin generation, was dose-dependently suppressed by rNAPc2. Prothrombin time also increased dose-dependently for 48 h after rNAPc2 administration (31, 32).

An additional multicenter, open-label phase II proof-of-concept study was initiated in August 2005 to assess the efficacy and safety of rNAPc2 to replace unfractionated heparin in patients being treated for ACS. The study will include 50-100 patients who can be randomized within 48 h of symptoms in cohorts of 25 patients each. The initial group of patients will receive a half-dose regimen of unfractionated heparin and 10 μg/kg of rNAPc2; the next group will receive no heparin and the same dose of rNAPc2 (12, 33).

Drug Interactions

A recent study was performed to evaluate if the combination of rNAPc2 with antagonists of gpIIb/IIIa could potentially show altered antiplatelet and anticoagulant effects. A lack of a significant effect on clotting and platelet function was observed, suggesting that concentrations of rNAPc2 up to 250 ng/ml will not produce substantial increases in the incidence of bleeding in clinical trials (34).

Source

Nuvelo, Inc. (US); licensed from Dendreon Corporation (US).

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