We are IntechOpen, the world's leading publisher of Open Access books Built by scientists, for scientists

6,300

Open access books available

172,000

International authors and editors

190M

Downloads

Our authors are among the

154

Countries delivered to

TOP 1%

most cited scientists

12.2%

Contributors from top 500 universities

WEB OF SCIENCE™

Selection of our books indexed in the Book Citation Index
in Web of Science™ Core Collection (BKCI)

Interested in publishing with us?
Contact book.department@intechopen.com

Numbers displayed above are based on latest data collected.
For more information visit www.intechopen.com

Prospective Efficacy and Safety of a Novel Bypassing Agent, FVIIa/FX Mixture (MC710) for Hemophilia Patients with Inhibitors

Kazuhiko Tomokiyo, Yasushi Nakatomi,
Takayoshi Hamamoto and Tomohiro Nakagaki
*Therapeutic Protein Product Research Department
The Chemo-Sero-Therapeutic Research Institute, Kaketsuken
Japan*

1. Introduction

Hemophilia A and B are hereditary bleeding disorders caused by a deficiency of coagulation factors VIII (FVIII) and IX (FIX), respectively. In substitution therapies using FVIII and FIX concentrates for the management of bleeding, the development of inhibitory antibodies is a serious complication in ~28% and ~7% of hemophilia A and B patients, respectively [1, 2].

Currently, two bypassing agents, plasma-derived activated prothrombin complex concentrates (APCC, FEIBA®) and recombinant activated factor VII (rFVIIa, NovoSeven®), are available for the management of bleeding in hemophilia patients with inhibitors including acquired hemophilia patients. Retrospective studies showed the efficacy of rFVIIa and APCC in 12~36 h in a standard administration regime is assessed to be 66~95% and 39~76%, respectively [3]. A recent comparative study seemed to indicate equivalence between rFVIIa and APCC [4]; however, a considerable number of patients experience treatment failure or insufficient efficacy.

In 2002, an anecdotal report suggested that the sequential administration of rFVIIa and prothrombin complex concentrate (PCC) to hemophilia patients with inhibitors in order to obtain a stronger hemostatic effect than with rFVIIa alone [5]. Further, it has been reported that the combination of rFVIIa and APCC appeared to confer beneficial hemostatic synergy in patients refractory to each individual therapy [6-8]. However, repeated infusion of APCC may cause an accumulation of prothrombin and factor X (FX), thereby increasing the risk of thrombosis. Moreover, a lack of suitable laboratory tests for monitoring hemostatic effects is a major concern with current bypassing therapies. As a consequence and in appreciation of the incomplete efficacy and safety of currently available bypassing agents, new drugs are in development, such as rFVIIa analogues featuring higher hemostatic potential [9], glycoPEGylated rFVIIa with a longer half-life than rFVIIa [10], and non-anticoagulant sulphated polysaccharides (AV513) [11].

In order to solve these problems, we searched for alternative factors for enhancing and promoting FVIIa's hemostatic activity, and found that a combination of plasma-derived FVIIa and FX (FVIIa/FX) may overcome the disadvantages of rFVIIa therapy for hemophilia patients with inhibitors. That combination allows improving APTT remarkably in the

patient plasma which is useful laboratory test for monitoring the hemostatic effect of hemophilia patients. An FVIIa/FX mixture, MC710, was designed as a dry-heated product prepared by mixing plasma-derived FVIIa and FX at a weight ratio of 1:10 under acidic conditions to suppress the generation of FXa.

A Phase I clinical study encompassing pharmacokinetics (PK), pharmacodynamics (PD), and safety has been completed. In this article, we outline the rationale for the combined use of FVIIa and FX, the manufacturing method of MC710, the treatment's prospective efficacy and safety, and in the final section, the results of Phase I clinical study in non-bleeding hemophilia patients with inhibitors.

2. Rationale for the combination of FVIIa and FX

2.1 Background

The blood coagulation system involves a so-called "cascade reaction" of enzymes and substrates which finally achieves the formation of a fibrin clot. In this reaction, Ca^{2+} and phospholipids (PL) assume the role of cofactors which remarkably enhance the affinity between enzymes and substrates, resulting in the promotion of a coagulation reaction at the site of injury.

FVIIa circulates in blood at a concentration of around 0.1 nM (3~5 ng mL⁻¹) in plasma [12, 13] with a much longer half life, 2~3 h, than other activated coagulation factors because of its zymogen-like conformation [14]. Tissue factor (TF) is a membrane glycoprotein expressed in various tissues and plays the role of a cofactor of FVIIa. Vascular TF is present in the adventitia, hidden from the circulating blood. When a vessel is injured, TF is exposed at the site. FVIIa binds to TF forming a stoichiometric complex (the initiator of the extrinsic pathway) and remarkably enhances catalytic activity to activate FIX and FX. FIXa forms a FIXa/FVIIIa/PL complex (FXase) and converts FX to FXa. Also, FXa forms a FXa/FVa/PL complex (prothrombinase) and converts prothrombin to thrombin. Thrombin activates FXI to form FXIa and promotes the activation of FIX (feedback activation of the intrinsic pathway), and converts the fibrinogen to a fibrin clot via limited proteolysis [15]. Platelets are essential for hemostasis. In the artery, circulating platelets in blood attach and adhere to the extracellular matrix (collagen) under the layer of endothelial cells at the site of damage using GPVI and integrin $\alpha_2\beta_1$ (GPIa/IIa), and adhere to the collagen via the interaction of VWF (von Willebrand factor) and GPIbIX, and finally aggregate through the interaction of $\alpha_{IIb}\beta_3$ (GPIIb/IIIa) and VWF/fibrinogen [16-19]. Through these processes, localized platelets are activated by thrombin or chemical mediators (ADP, *et al*) released from activated platelet granules. The PL membrane essential for thrombin generation is mainly supplied by the activated platelets.

In the hemostasis of hemophilia patients with inhibitors against FIX or FVIII, the intrinsic pathway is completely blocked. Therefore, it is necessary to enhance the potential for coagulation based on the extrinsic pathway, the activation of FX by FVIIa, followed by the activation of prothrombin to form thrombin.

In 1996, rFVIIa therapy was launched to give a super-physiological concentration to the patient plasma to enhance the extrinsic pathway of hemophilia patients with inhibitors. However, the mechanism of the bypassing activity of rFVIIa is controversial due to the requirement for a high dose (60~120 $\mu\text{g kg}^{-1}$), which gives a high FVIIa concentration in plasma (0.5~1.0 $\mu\text{g mL}^{-1}$) [20].

There are two hypotheses to elucidate the requirement for the high dose (**Fig. 1-A**). The first is “TF-dependent FX activation”. It was reported that rFVIIa-induced thrombin generation in the presence of a low concentration of TF and in the absence of FVIII is inhibited by physiological concentrations of FVII, and at least 10 nM (0.5 µg mL⁻¹) of rFVIIa is required to overcome the inhibition by FVII in order to induce the bypassing activity [21, 22]. The second is “TF-independent FX activation”. It was reported that 5 nM (0.25 µg mL⁻¹) of rFVIIa could convert FX to FXa on the surface of activated platelets, leading to thrombin generation independent of TF [23]. Clinical experience has suggested that the dose or blood level of rFVIIa required for hemostasis in any given hemophilia patient with inhibitors is not always predictable. Differences in platelet procoagulant properties could influence the response to a high-dose of rFVIIa [24, 25]. Also, it has been reported that rFVIIa binds to GPIbIX on activated platelets and localizes at the site of injury [26]. Recently, mega-dose therapy with an injection of rFVIIa (270 µg kg⁻¹) has been applied to the control of bleeding to diminish repeated administrations in 2~3 h and raise the hemostatic potential of rFVIIa by increasing the C_{max} in plasma above the standard dose [27, 28].

The exact mechanism of rFVIIa is not clear at present, but its potential for FXa and thrombin generation and subsequent fibrin clot formation will be essential to obtain a hemostatic effect with rFVIIa, in either a TF-dependent or TF-independent manner.

2.2 Rationale for the combined use of FVIIa and FX

It was reported that TF binds to FX or FIX as well as to FVIIa and this ternary complex of enzyme/cofactor/substrate (FVIIa/TF/ FIX or FX) is the real trigger of the extrinsic pathway to generating FIXa or FXa and the subsequent generation of thrombin [29-31]. However, it was demonstrated that FIX is a much better substrate for FVIIa-TF than FX [32]. We investigated the kinetic parameters of FVIIa-mediated FX activation under several conditions. The results of the kinetic analysis are shown in **Table 1**. K_m values in the presence of PL and relipidated TF were 180 ± 70 nM and 160 ± 60 nM, while k_{cat} values were 0.38 ± 0.09 s⁻¹ and 11.5 ± 4.7 s⁻¹, respectively, similar to values published previously [32]. The FX level in normal plasma is 8~12 µg mL⁻¹ (140~210 nM) [34], and the K_m values were within this range; therefore, it was suggested that the FX concentration in plasma might not be sufficient to achieve the generation of FXa by FVIIa required for hemostasis (**Fig. 1-B**). These results indicate that a higher concentration of FX is required to enhance the catalytic efficacy of FVIIa and to complete coagulation in the plasma of hemophilia patients.

Assay conditions	Concentration	K _m µM	k _{cat} s ⁻¹	k _{cat} /K _m s ⁻¹ µM ⁻¹
Ca ²⁺	5 mM	72.4	0.000135	0.00000187
Ca ²⁺ /PCPS ^{a)}	5 mM/10 µM	0.18±0.07	0.38±0.09	2.20±0.34
Ca ²⁺ /PCPS/TF	5 mM/10 µM/100 pM	0.16±0.06	11.5±4.7	73.1±14.3
Ca ²⁺ /activated platelets ^{b)}	5 mM/1.5×10 ⁵ cells µL ⁻¹	1.72±0.4	0.77±0.21	0.48±0.19

The activation of FX was carried out in the presence or absence of PL (10 µM) and relipidated TF (100 pM TF/10 µM PL) in a solution containing 5 mM CaCl₂[33].

a) Reconstituted phosphatidylcholine and phosphatidylserine vesicles (PCPS; weight ratio 7:3) were used as phospholipids.

b) Platelets were activated by the thrombin receptor activation peptide (TRAP).

Table 1. Steady-state kinetics of FX’s activation by FVIIa

(A) FVIIa induced FXa generation. The activation of FX is essential for FVIIa to generate bypassing activity. For the elucidation of the requirement for high rFVIIa concentrations ($>1 \mu\text{g mL}^{-1}$ in plasma) in rFVIIa therapy, there are two hypotheisies: “TF-dependent FX activation” [21, 22] and “TF-independent FX activation” [23-26] (refer to the text for details). (B) Kinetic analysis of FVIIa to FX. In the activation of FX by FVIIa, the activation rate (y-axis) increases depending on the substrate (FX) concentration (x-axis). As shown in Table 1, K_m values in the presence of PL and relipidated TF were in the range of FX levels in normal plasma (140~210 nM), and the K_m value in activated platelets is far above that range. In the extrinsic pathway, the increase of FX concentration in plasma two or three times ($2\sim 3 \times K_m$) might facilitate the increase of the FX activation rate, and promote the formation of FVIIa/TF/FX complex.

Fig. 1. Advantage of co-administration of FVIIa and FX

The results of the kinetic analysis were consistent with those of the thrombin generation (TG) assay (Fig. 2). TG assay using a fluorosubstrate (Z-G-G-R-MCA) specific for thrombin was developed by Hemker et al [35]. In this assay system, thrombin generation is analyzed in the following three steps [36, 37]:

1. Initiation: initiation of the cascade reaction to start thrombin generation.
2. Propagation: explosive thrombin production.
3. Termination: attenuation of thrombin generation.

The TG assay is used to analyze the clinical efficacy of rFVIIa and APCC [38]. The TG parameters are lag time (time to initial thrombin formation (min)), peak thrombin level (nM), time to peak (ttPeak) (min), and endogenous thrombin potential (ETP) (nM min) [39]. We used this assay to examine the hemostatic potential of the combination of FVIIa and FX. The thrombin-generating potential of hemophilic plasma was raised by increasing the concentration of FX added to the plasma in the range of $2.5\sim 20 \mu\text{g mL}^{-1}$ without the addition of FVIIa (Figs. 2A-a and 2B-a). Further, the combination of FVIIa (0.25 and $1.0 \mu\text{g mL}^{-1}$) and FX ($2.5\sim 20 \mu\text{g mL}^{-1}$) gave more thrombin-generating potential to the plasma than did FVIIa alone, resulting in a shortening of the ttPeak and an increase in the peak thrombin level (Figs. 2A-b and -c, and 2B-b and -c) [40].

It has been estimated that the half-life of FVIIa is around 3 h and that of FX is 24~56 h [41-43]. Following the simultaneous administration of FVIIa and FX, the amount of FVIIa should

decrease faster than that of FX, but a high FX level might help to generate bypassing activity which results in longer-lasting hemostatic potential than FVIIa alone. This idea was proven in our previous experiment using a monkey acquired hemophilia B model in which the hemostatic efficacy of FVIIa/FX (co-administration of FVIIa ($80\text{ }\mu\text{g kg}^{-1}$) and FX ($800\text{ }\mu\text{g kg}^{-1}$)) and FVIIa alone ($80\text{ }\mu\text{g kg}^{-1}$) was compared by measuring thromboelastography (TEG). As shown in Fig. 3, administration of FVIIa/FX remarkably normalized TEG parameters. Even 6 h after its administration, FVIIa/FX had hemostatic potential above that immediately after the administration of FVIIa alone [44].

TG in plasma of a hemophilia A patient with inhibitors (FVIII INP; 101 BU mL^{-1}) or FIX-depleted plasma (FIX-DP) at various concentrations of FVIIa and FX with relipidated TF. Panels a~c of A (FVIII INP) and B (FIX-DP): FVIIa 0, 0.25, and $1.0\text{ }\mu\text{g mL}^{-1}$. Added FX concentration in the plasma from top to bottom in each panel: $0\text{ }\mu\text{g mL}^{-1}$, —; $2.5\text{ }\mu\text{g mL}^{-1}$, —; $5.0\text{ }\mu\text{g mL}^{-1}$, —; $10\text{ }\mu\text{g mL}^{-1}$, —; $20\text{ }\mu\text{g mL}^{-1}$, —. 16.7 pM TF and $0.83\text{ }\mu\text{M}$ PL were used for the assay.

Fig. 2. Changes in TG profiles induced by FX on FVIIa in hemophilic plasma⁽⁴⁰⁾

APTT is used for monitoring the management of bleeding or determining the supplemental level of FVIII or FIX concentrate in hemophilia patients, but not in therapy using bypassing agents because of the poor improvement. We reported that more than $5\text{ }\mu\text{g mL}^{-1}$ of FVIIa alone is required to reduce APTT in hemophilic plasma to levels equivalent to those after replacement-therapy (10 % of FVIII or FIX activity in hemophilic plasma); on the other hand, the mixture of FX and FVIIa caused a significant improvement of APTT in a concentration-dependent manner. In plasma containing $0.5\sim 1.5\text{ }\mu\text{g mL}^{-1}$ of FVIIa (obtained after intravenous rFVIIa administration at standard doses) when FX is added at $5\sim 15\text{ }\mu\text{g mL}^{-1}$, the poor coagulant activity in hemophilic plasma is remarkably improved to levels achieved

with replacement therapy [44]. Therefore, a mixture ratio 1: 10 of FVIIa to FX in MC710 was designed to optimize the bypassing effect of FVIIa ($0.5\sim1.5\text{ }\mu\text{g mL}^{-1}$) in plasma.

A hemophilia B model using a cynomolgus monkey was produced by the administration of goat anti-FIX antibodies to be in < 5% of FIX activity. FVIIa ($80\text{ }\mu\text{g/kg}$) was administered to the monkey and Thromboelastography (TEG) was measured pre-administration, and 6 h and 12 h post-administration. Also, FVIIa ($80\text{ }\mu\text{g/kg}$) and FX ($800\text{ }\mu\text{g/kg}$) were continuously injected into the monkey. The TEG patterns are shown in the figure and $r + k$ values are described on the right side of each pattern. ND means “not detected”.

Fig. 3. Changes in TEG patterns in the monkey acquired hemophilia B model pre/post injection of FVIIa or FVIIa/FX⁽⁴⁴⁾

3. Outline of the manufacture of the FVIIa/FX mixture (MC710)

MC710 is a lyophilized mixture of FVIIa and FX prepared from pooled human plasma. FVII and FX are vitamin K-dependent proteins containing a strongly negatively charged amino acid, γ -carboxy-glutamic acid. The purification of those proteins takes into account specific properties such as high acidity and Ca^{2+} -dependent conformational change. The conversion of FVII to FVIIa is a key process in the production of the FVIIa preparation. In 1986, Bjoern *et al.* reported that rFVII is autocatalytically activated to rFVIIa on an anion exchange column [45]. On an industrial scale, the activation of FVII on an anion exchange resin is very useful because it does not require any other proteases to activate FVII such as FXa, FIXa, and FXIIa. In the production of plasma-derived FVIIa, FVII is converted to FVIIa with the following two steps to achieve high recovery and quality: (1) partial activation on anion exchange resin and, (2) further activation in the solution after eluting from the resin [46]. A flow diagram of the preparation of MC710 is shown in Fig. 4. In the first purification step, a crude

vitamin K-dependent protein fraction is extracted from cryoprecipitate-poor plasma using anion exchange chromatography. Next, this fraction is applied to an immunoaffinity chromatography column containing gels bound with Ca²⁺-dependent anti-FVII or anti-FX monoclonal antibody as a ligand. The FVII or FX fraction eluted with a buffer containing EDTA is treated in solvent and detergent (0.3% TNBP and 1% polysorbate 80) for virus inactivation. After the treatment, the FVII fraction is applied to DEAE Sepharose-FF to obtain partially activated FVIIa and the eluted FVII/FVIIa mixture is completely converted to FVIIa in the solution. The FX fraction eluted from the immunoaffinity chromatography

FVII and FX are purified using immunoaffinity gel coupled with anti- FVII and -FX antibodies, respectively. Purified fractions are treated with solvent and detergent (0.3% TNBP and 1% polysorbate 80) for inactivation of the enveloped virus, and purified samples are filtered with a nano-filter of 15-nm pore size (Planova® 15N) for virus elimination. The specific activity of the prepared FVIIa and FX was 48.1 ± 0.5 IU/μg (n=5) and 159.1 ± 4.4 IU/mg (n=6), respectively. After mixing FVIIa and FX with the stabilizers, the solution is dispensed to the vials and lyophilized, then heated at 65°C for 96 h.

Fig. 4. Outline of manufacturing methods of MC710

column is further purified using anion exchange and hydrophobic interaction chromatographies. These purified samples are filtered with a nano-filter (Planova® 15N) of 15-nm pore size for virus elimination. The concentrated FVIIa and FX are mixed at a weight ratio of 1:10 under acidic conditions of pH5.5~5.9 for suppression of FXa generation, and then lyophilized. Finally, the lyophilized FVIIa and FX mixture is dry-heated at 65°C for 96 h for virus elimination. MC710 is formulated with FVIIa (0.6 mg mL⁻¹), FX (6 mg mL⁻¹), antithrombin (1.0 U mL⁻¹), and human serum albumin (2.0%) after reconstitution [40].

4. Prospective efficacy and safety of MC710

4.1 APTT and PT waveform analysis

APTT waveforms are used to analyze the overall process of fibrin clotting by measuring the turbidity and calculating the coagulation rate (dT/dt) and coagulation acceleration (second derivative of transmittance and time; d^2T/dt^2). It was reported that these parameters are useful for the diagnosis of DIC [47, 48]. Recently, Shima reported that APTT waveforms are applicable to the quantification of low levels of FVIII (<1 U dL⁻¹) on the basis of the correlation of the FVIII activity with coagulation acceleration, and the waveform profile formed by rFVIIa was different from that for normal plasma [49]. In our analysis, MC710 with a FVIIa concentration of 1 µg mL⁻¹ (the dose and concentration in plasma of MC710 are expressed as FVIIa amounts) exhibited greater clotting ability than 1 µg mL⁻¹ FVIIa alone in hemophilia A patient plasma with inhibitors and FIX-deficient plasma samples (**Figs. 5A-a and 5B-a**). Coagulation acceleration showed that MC710 at above 1-2 µg mL⁻¹ possessed a greater ability to shorten APTT and to induce accelerated clotting than did the same concentration of rFVIIa (**Figs. 5A-b and 5B-b**). Parameters for plasma from hemophilia A patients with inhibitors in the presence of 1 U mL⁻¹ APCC were similar to those in the presence of 1 µg mL⁻¹ MC710 (**Figs. 5A-a and 5A-b**). On the other hand, PT and its clot formation acceleration did not show any difference among the three agents (**data not shown**).

4.2 TG assay

The thrombin generation of hemophilia A patient plasma with inhibitors and FIX-depleted plasma was measured at various concentrations of rFVIIa, MC710, and APCC. MC710 (0.1~8.0 µg mL⁻¹) and APCC (0.25~2.0 U mL⁻¹) exhibited conspicuous concentration-dependent changes in thrombin generation profiles (**Figs. 6A-b, 6B-b, and 7B-a**). Reportedly, a mega-dose (270 µg kg⁻¹ b.w.) of rFVIIa can achieve rFVIIa concentrations of 3~5 µg mL⁻¹ in hemophilic plasma [27, 28]; however, the thrombin-generating potential of rFVIIa in plasma did not change at over 1 µg mL⁻¹ of rFVIIa (**Figs. 6A-a and 6B-a**).

To evaluate the TF-specificity of the agents, thrombin generation in plasma of a hemophilia patient with inhibitors was measured in the presence or absence of relipidated TF or PL at various concentrations of MC710 and APCC, and 1.0 µg mL⁻¹ of rFVIIa (**Figs. 7A-a~c and 7B-a~c**). The 0.1 µg mL⁻¹ of MC710 and 0.25 U mL⁻¹ of APCC showed a greater thrombin generation potential than 1.0 µg mL⁻¹ of rFVIIa (**Figs. 7A-a and 7B-a**). MC710 showed lower thrombin-generating potential than did APCC in the absence of TF or PL (**Figs. 7A-b and -c, and 7B-b and -c**). These results suggest that MC710 has a relatively high specificity for TF compared to APCC.

APTT waveforms of plasma from a hemophilia A patient with inhibitors (FVIII INP; 140 BU mL⁻¹) and FIX-depleted plasma (FIX-DP) at various concentrations of the bypassing agents. Panels A-a and A-b: APTT and coagulation acceleration of FVIII INP containing rFVIIa (NovoSeven®) (0.1~8.0 μg mL⁻¹, □), MC710 (0.1~8.0 μg mL⁻¹, ■), and APCC (FEIBA®) (0.25~4.0 U mL⁻¹). Panels B-a and B-b: APTT and coagulation acceleration of FIX-DP containing rFVIIa (0.1~8.0 μg mL⁻¹, □), MC710 (0.1~8.0 μg mL⁻¹, ■). Normal plasma ("Normal") or FIX-DP supplemented with FIX at 1 U mL⁻¹ was used as a control. The MC710 concentration is denoted by the FVIIa concentration in each panel.

Fig. 5. Changes in APTT waveforms induced by rFVIIa, APCC, and MC710(40)

TG in plasma of a hemophilia A patient on inhibitors (FVIII INP; 70 BU mL⁻¹) and FIX-depleted plasma (FIX-DP) at various concentrations of the bypassing agents with relipidated TF. Panels A-a and -b: TG of FVIII INP; rFVIIa (NovoSeven®) (A-a, 0.1~8 µg mL⁻¹) and MC710 (A-b, 0.1~8.0 µg mL⁻¹). Panels B-a and -b: TG of FIX-DP, rFVIIa (B-a, 0.1~8.0 µg mL⁻¹), MC710 (B-b, 0.1~8.0 µg mL⁻¹). The gray lines signify the results in normal plasma ("Normal") for A-a and -b and FIX-DP supplemented with FIX 1.0 U mL⁻¹ for B-a and B-b used as control plasma. The MC710 concentration is denoted by the FVIIa concentration in each panel. In the TG assay, 16.7 pM TF and 0.83 µM PL were used. Each TG profile represents the results of three experiments.

Fig. 6. Changes in TG profiles induced by rFVIIa and MC710⁽⁴⁰⁾

TG in plasma of a hemophilia A patient on inhibitors (38 BU mL^{-1}) with relipidated TF or phospholipids or without relipidated TF at various concentrations of the bypassing agents. Panels A- and B-a~c: a) addition of relipidated TF, b) addition of phospholipids, c) no addition of relipidated TF at the concentrations of MC710 ($0.1\sim2.0 \mu\text{g mL}^{-1}$; panels A a~c) and APCC (FEIBA®) ($0.25\sim2.0 \text{ U mL}^{-1}$; panels B a~c). TG in the presence of rFVIIa (NovoSeven®) ($1.0 \mu\text{g mL}^{-1}$) is inserted in each panel. The MC710 concentration is denoted by the FVIIa concentration in each panel. Each TG profile represents the results of three experiments. In the TG assay, 16.7 pM TF and $0.83 \mu\text{M}$ PL were used.

Fig. 7. Changes in TG profiles induced by APCC and MC710 with or without TF⁽⁴⁰⁾

4.3 Thrombogenic test using monkeys

It was reported that APCCs might induce thrombotic complications such as disseminated intravascular coagulation (DIC) and acute myocardial infarction [50-52]. As the clearance of FX is much slower than that of FVIIa, repeated administrations of MC710 might induce the accumulation of FX in the blood raising concerns over safety regarding DIC or other thrombotic events. Therefore, it is important to confirm the safety of repeated administrations of MC710 alone and in combination with other bypassing agents. We performed multiple injections of MC710 (4 injections of 120 $\mu\text{g kg}^{-1}$ every 8 h (as FVIIa dose)), and rFVIIa (one injection of 90 $\mu\text{g kg}^{-1}$ and 2 injections of 120 $\mu\text{g kg}^{-1}$ every 2 h) or APCC (3 infusions of 100 U kg^{-1} every 12 h) at 8 h after the administration of MC710 (120 $\mu\text{g kg}^{-1}$) into the monkeys, and compared the DIC parameter changes with those of APCC (4 infusion of 100 U kg^{-1} every 12 h) (Fig. 8). No serious or severe event was observed in any monkey or in any group, and the fibrinogen level and platelet counts did not change. However, the FDP (fibrinogen degraded products) level increased in all groups and the rate of increase was lower in the group repeatedly administered MC710 than that repeatedly administered APCC (See the legend in Fig. 8). These results suggest the thrombogenic risk from the repeated administration of MC710 is equal to or lower than that of repeated administration of APCC.

Thrombogenicity of MC710 was compared to APCC (FEIBA®) using cynomolgus monkeys. The experimental design is described in the figure. Schemes a)~c) show the time courses of injections or infusions of rFVIIa (NovoSeven®) and APCC after 120 $\mu\text{g kg}^{-1}$ of MC710. MC710 dose is denoted as FVIIa dose. Scheme d) shows the time course of repeated infusions of APCC. The experiment was performed using three monkeys in each group. At pre-administrations FDP level was $0.44 \pm 0.24 \text{ ng mL}^{-1}$ ($n=12$) and at 30 min after the final administration of the agents a), b), c), and d) were 3.37 ± 3.59 , 5.87 ± 2.64 , 2.93 ± 0.42 , and $8.57 \pm 2.17 \text{ ng mL}^{-1}$ ($n=3$), respectively.

Fig. 8. Design for the thrombogenic test for MC710

5. Summary of phase I clinical study

5.1 Outline of the trial

Phase I clinical study of MC710 has been completed. In this study, PK and PD parameters and the safety of single doses of MC710 were investigated in 11 hemophilia patients with inhibitors in a non-bleeding state. A total of 25 administrations of MC710 were made to the subjects (7 hemophilia A patients with inhibitors and 4 hemophilia B patients with inhibitors) at 5 doses

of MC710 (20, 40, 80, 100 and 120 $\mu\text{g kg}^{-1}$ (as FVIIa dose)) in addition to the administrations of rFVIIa 120 $\mu\text{g kg}^{-1}$, and APCC 50 U kg^{-1} or 75 U kg^{-1} as active controls [53].

5.2 Pharmacokinetic analysis

PK parameters were calculated based on FVII:C, FVII:Ag, FX:C and FX:Ag levels. As shown in Figs. 9A-D, those levels rapidly increased after administration of MC710. FVII:C and FVII:Ag levels returned to pre-administration values during 12 to 24 h after the administration, and increased levels of FX:C and FX:Ag persisted in the blood until 48 h after the administration of MC710 at 80 $\mu\text{g kg}^{-1}$ or more. The mean $t_{1/2}$ of FVII:C and FVII:Ag in the MC710-infused group was 2.1~3.4 h and 3.5~4.9 h, respectively, and that of FX:C and FX:Ag was 20.2~23.2 h and 22.8~27.5 h, respectively, shorter than reported values ($t_{1/2}$ of FX:C 24~56 h [42, 43]). On the other hand, the recovery of FVII:C and FVII:Ag in the MC710-infused group was 70.7~90.7% and 50.0~92.0%, respectively, and that of FX:C and FX:Ag was 98.7~120.9% and 94.0~109.4%, respectively. PK parameters of FVIIa were similar to previously reported values for rFVIIa [41].

Time-dependent changes in the pharmacokinetics of FVII:C (Panel A), FVII:Ag (Panel B), FX:C (Panel C) and FX:Ag (Panel D) are shown. Enlarged figures of the changes in FVII:C and FVII:Ag until 6 hr after administration are shown in the right upper corner of each graph. The mark represents the mean $\pm$ SD. MC710 doses are denoted by the following color symbols: 20 $\mu\text{g kg}^{-1}$, (-●-); 40 $\mu\text{g kg}^{-1}$, (-●-); 80 $\mu\text{g kg}^{-1}$, (-●-); 100 $\mu\text{g kg}^{-1}$, (-●-); 120 $\mu\text{g kg}^{-1}$, (-●-). MC710 doses are denoted as FVIIa dose.

Fig. 9. Changes in pharmacokinetic parameters after the administration of MC710 to hemophilia patients with inhibitors⁽⁵³⁾

5.3 Pharmacodynamic analysis

APTT and PT were measured as PD parameters. APTT, prolonged 120 sec or more before administration, improved in a dose-dependent manner after administration of MC710, and the effect persisted for 12 h at all doses (**Fig. 10A**). At MC710 doses of more than 100 $\mu\text{g kg}^{-1}$,

Time-dependent changes in APTT (Panel A) and PT (Panel B) are shown. The normal ranges for healthy individuals (---) for APTT were defined as 42.5 (upper limit) and 23.5 (lower limit) sec and for PT as 12.8 (upper limit) and 9.9 (lower limit) sec. The mark represents the mean $\pm$ SD. MC710, rFVIIa (NovoSeven®) and APCC (FEIBA®) doses are denoted by the following color symbols: MC710 (as FVIIa dose); 20 $\mu\text{g kg}^{-1}$, (-●-); 40 $\mu\text{g kg}^{-1}$, (-●-); 80 $\mu\text{g kg}^{-1}$, (-●-); 100 $\mu\text{g kg}^{-1}$, (-●-); 120 $\mu\text{g kg}^{-1}$, (-●-); rFVIIa; 120 $\mu\text{g kg}^{-1}$, (-▲-); APCC; 50 U kg^{-1} , (-■-); 75 U kg^{-1} , (-■-).

Fig. 10. Changes in pharmacodynamic parameters after the administration of MC710 to hemophilia patients with inhibitors⁽⁵³⁾

the APTT was especially close to the normal range. Even 6 h after the administration of more than 100 $\mu\text{g kg}^{-1}$ of MC710, the APTT was shorter than that immediately after the administration of 120 $\mu\text{g kg}^{-1}$ of rFVIIa and 75 U kg^{-1} of APCC. It is expected that from the evaluation based on the level of improvement in APTT, the hemostatic effect immediately after the administration of MC710 at over 100 $\mu\text{g kg}^{-1}$ might be equivalent to that of FVIII or FIX replacement therapy (replacement level 20 to 50% of these factors).

The PT reached approximately 6 sec (the determination limit) after administration of all doses of MC710 except for 20 $\mu\text{g kg}^{-1}$ and remained at that level for up to 2 h. At 6 h after the administration of 80, 100 and 120 $\mu\text{g kg}^{-1}$ of MC710, the PT was shorter than that after the administration of 120 $\mu\text{g kg}^{-1}$ of rFVIIa. The reduction in PT persisted for 12 h at all doses (**Fig. 10B**). The PT after the administration of 40, 80, 100 and 120 $\mu\text{g kg}^{-1}$ of MC710 was shorter than that for 75 U kg^{-1} of APCC.

5.4 DIC and other safety concerns

TAT and F1+2 were increased after the administration of MC710 indicating the activation of prothrombin in blood flow; however, similar increases were also observed after the administration of rFVIIa and APCC [54, 55]. No serious or severe adverse events were observed within 4 weeks after the administration of MC710 and no subject discontinued treatment due to an adverse event. Also, no clinical symptoms or changes in laboratory tests (platelet count, fibrinogen, D-dimer) indicating a hypercoagulable state such as DIC were detected (**data not shown**). In addition, the results of virologic and serologic tests confirmed

that no subject developed a new viral antigen or produced a new antibody after the administration of MC710.

6. Conclusion and future perspectives

In this review, we described the rationale for the combined use of FVIIa and FX, the manufacturing process of FVIIa/FX mixture, MC710, and the treatment's prospective efficacy and safety. We also outlined the results of a Phase I clinical study. In the study, PK and PD parameters changed in a dose-dependent manner after the administration of MC710 and the changes in the PD parameters (APTT and PT) were equal to or greater than those in rFVIIa and APCC. Furthermore, MC710 was safely administered at doses of up to 120 $\mu\text{g kg}^{-1}$ and no serious or severe adverse events, including DIC, were observed.

It was recently reported that the combination of APCC and rFVIIa is safe and effective in the treatment of bleeding that is unresponsive to monotherapy [56]. This report supports our hypothesis that the FVIIa/FX mixture, MC710, would be a more potent bypassing agent than clinically available bypassing agents. Phase II clinical studies in hemophilia patients with inhibitors who are hemorrhaging have been completed in Japan and MC710 is expected to be used as an alternative to APCC and rFVIIa in the near future.

7. References

- [1] Wight J, Paisley S. The epidemiology of inhibitors in hemophilia A: a systematic review. *Hemophilia* 2003;9:418-435.
- [2] DiMichele D. Inhibitor development in hemophilia B: an orphan disease in need of attention. *Br J Haematol* 2007;138:305-15.
- [3] Treur MJ, McCracken F, Heeg B, Joshi AV, Botteman MF, De Charro F, Van Hout B. Efficacy of recombinant activated factor VII vs. activated prothrombin complex concentrate for patients suffering from hemophilia complicated with inhibitors: a Bayesian meta-regression. *Hemophilia* 2009;15:420-36.
- [4] Astermark J, Donfield SM, DiMichele DM, Gringeri A, Gilbert SA, Waters J, Berntorp E; FENOC Study Group. A randomized comparison of bypassing agents in hemophilia complicated by an inhibitor: the APCC RFVIIa Comparative (FENOC) Study. *Blood* 2007;109:546-51.
- [5] Key NS, Christie B, Henderson N, Nelsestuen GL. Possible synergy between recombinant factor VIIa and prothrombin complex concentrate in hemophilia therapy. *Thromb Haemost* 2002;88:60-5.
- [6] Schneiderman J, Rubin E, Nugent DJ, Young G. Sequential therapy with activated prothrombin complex concentrates and recombinant FVIIa in patients with severe hemophilia and inhibitors: update of our previous experience. *Haemophilia* 2007;13:244-8.
- [7] Economou M, Teli A, Tzantzaroudi A, Tsatra I, Zavitsanakis A, Athanassiou-Metaxa M. Sequential therapy with activated prothrombin complex concentrate (APCC) and recombinant factor VIIa in a patient with severe hemophilia A, inhibitor presence and refractory bleeding. *Haemophilia* 2008;14:390-1.
- [8] Livnat T, Martinowitz U, Zivelin A, Seligsohn U. Effects of factor VIII inhibitor bypassing activity (APCC), recombinant factor VIIa or both on thrombin generation in normal and hemophilia A plasma. *Haemophilia* 2008;14:782-6.

- [9] Møss J, Scharling B, Ezban M, Møller Sørensen T. Evaluation of the safety and pharmacokinetics of a fast-acting recombinant FVIIa analogue, NN1731, in healthy male subjects. *J Thromb Haemost* 2009;7:299-305.
- [10] Stennicke HR, Ostergaard H, Bayer RJ, Kalo MS, Kinealy K, Holm PK, Sørensen BB, Zopf D, Bjørn SE. Generation and biochemical characterization of glycoPEGylated factor VIIa derivatives. *Thromb Haemost* 2008;100:920-8.
- [11] Prasad S, Lillicrap D, Labelle A, Knappe S, Keller T, Burnett E, Powell S, Johnson KW. Efficacy and safety of a new-class hemostatic drug candidate, AV513, in dogs with hemophilia A. *Blood* 2008;111:672-9.
- [12] Morrissey JH, Macik BG, Neuenschwander PF, Comp PC. Quantitation of activated factor VII levels in plasma using a tissue factor mutant selectively deficient in promoting factor VII activation. *Blood* 1993;81:734-44.
- [13] Wildgoose P, Nemerson Y, Hansen LL, Nielsen FE, Glazer S, Hedner U. Measurement of basal levels of factor VIIa in hemophilia A and B patients. *Blood* 1992;80:25-8.
- [14] Higashi S, Matsumoto N, Iwanaga S. Molecular mechanism of tissue factor-mediated acceleration of factor VIIa activity. *J Biol Chem* 1996;271:26569-74.
- [15] Davie EW, Fujikawa K, Kisiel W. The coagulation cascade: initiation, maintenance, and regulation. *Biochemistry* 1991;30:10363-70.
- [16] Goto S, Salomon DR, Ikeda Y, Ruggeri ZM. Characterization of the unique mechanism mediating the shear-dependent binding of soluble von Willebrand factor to platelets. *J Biol Chem* 1995;270:23352-61.
- [17] Moroi M, Jung SM, Nomura S, Sekiguchi S, Ordinas A, Diaz-Ricart M. Analysis of the involvement of the von Willebrand factor-glycoprotein Ib interaction in platelet adhesion to a collagen-coated surface under flow conditions. *Blood* 1997;90:4413-24.
- [18] Tomokiyo K, Kamikubo Y, Hanada T, Araki T, Nakatomi Y, Ogata Y, Jung SM, Nakagaki T, Moroi M. Von Willebrand factor accelerates platelet adhesion and thrombus formation on a collagen surface in platelet-reduced blood under flow conditions. *Blood* 2005;105:1078-84.
- [19] Kulkarni S, Dopheide SM, Yap CL, Ravanat C, Freund M, Mangin P, Heel KA, Street A, Harper IS, Lanza F, Jackson SP. A revised model of platelet aggregation. *J Clin Invest* 2000;105:783-91.
- [20] Hedner U: Recombinant activated factor VII as a universal haemostatic agent. *Blood Coagul Fibrinolysis* 1998;Suppl 1:147-52.
- [21] van 't Veer C, Golden NJ, Mann KG: Inhibition of thrombin generation by the zymogen factor VII: implications for the treatment of hemophilia A by factor VIIa. *Blood* 2000;95:1330-1335.
- [22] Butenas S, Brummel KE, Branda RF, Paradis SG, Mann KG. Mechanism of factor VIIa-dependent coagulation in hemophilia blood. *Blood* 2002;99:923-30.
- [23] Monroe DM, Hoffman M, Oliver JA, Roberts HR: Platelet activity of high-dose factor VIIa is independent of tissue factor. *Br J Haematol* 1997;99:542-47.
- [24] Monroe DM, Hoffman M, Oliver JA, Roberts HR: A possible mechanism of action of activated factor VII independent of tissue factor. *Blood Coagul Fibrinolysis* 1998;Suppl 1:15-20.
- [25] Hoffman M, Monroe DM. The action of high-dose factor VIIa (FVIIa) in a cell-based model of hemostasis. *Semin Hematol* 2001;38(Suppl 12):6-9.
- [26] Weeterings C, de Groot PG, Adelmeijer J, Lisman T. The glycoprotein Ib-IX-V complex contributes to tissue factor-independent thrombin generation by recombinant factor VIIa on the activated platelet surface. *Blood* 2008;112:3227-33.

- [27] Kenet G, Lubetsky A, Luboshitz J, Martinowitz U. A new approach to treatment of bleeding episodes in young hemophilia patients: a single bolus mega dose of recombinant activated factor VII (rFVIIa). *J Thromb Haemost* 2003;1:450-5.
- [28] Kavakli K, Makris M, Zulfikar B, Erhardttsen E, Abrams ZS, Kenet G; RFVIIa trial (F7HAEM-1510) investigators. Home treatment of haemarthroses using a single dose regimen of recombinant activated factor VII in patients with hemophilia and inhibitors. A multi-centre, randomised, double-blind, cross-over trial. *Thromb Haemost* 2006;95:600-5.
- [29] Chen SW, Pellequer JL, Schved JF, Giansily-Blaizot M. Model of a ternary complex between activated factor VII, tissue factor and factor IX. *Thromb Haemost* 2002;88:74-82.
- [30] Kittur FS, Manithody C, Rezaie AR. Role of the N-terminal epidermal growth factor-like domain of factor X/Xa. *J Biol Chem* 2004;279:24189-96.
- [31] Ndonwi M, Broze G Jr, Bajaj SP. The first epidermal growth factor-like domains of factor Xa and factor IXa are important for the activation of the factor VII-tissue factor complex. *J Thromb Haemost* 2005;3:112-8.
- [32] Komiyama Y, Pedersen AH, Kisiel W. Proteolytic activation of human factors IX and X by recombinant human factor VIIa: effects of calcium, phospholipide and tissue factors. *Biochemistry* 1990;29:9418-25.
- [33] Turecek PL, Varadi K, Keil B, Negrier C, Berntorp E, Astermark J, Bordet JC, Morfini M, Linari S, Schwarz HP. Factor VIII inhibitor-bypassing agents act by inducing thrombin generation and can be monitored by a thrombin generation assay. *Pathophysiol Haemost Thromb* 2003;33:16-22.
- [34] Fair DS, Plow EF, Edgington TS. Combined functional and immunochemical analysis of normal and abnormal human factor X. *J Clin Invest* 1979;64:884-94.
- [35] Hemker HC, Giesen PL, Ramjee M, Wagenvoord R, Beguin S. The thrombogram: monitoring thrombin generation in platelet-rich plasma. *Thromb Haemost* 2000;83:589-91.
- [36] Castoldi E, Rosing J. Thrombin generation tests. *Thromb Res* 2011;127(Suppl 3):S21-5.
- [37] Brummel-Ziedins KE, Vossen CY, Butenas S, Mann KG, Rosendaal FR. Thrombin generation profiles in deep venous thrombosis. *J Thromb Haemost* 2005;3:2497.
- [38] Váradi K, Negrier C, Berntorp E, Astermark J, Bordet JC, Morfini ML-Linari S, Schwarz HP, Turecek PL. Monitoring the bioavailability of APCC with a thrombin generation assay. *J Thromb Haemost* 2005;3:2497-505.
- [39] Hemker HC, Al Dieri R, De Smedt E, Béguin S. Thrombin generation, a function test of the haemostatic-thrombotic system. *Thromb Haemost* 2006;96:553-61.
- [40] Nakatomi Y, Nakashima T, Gokudan S, Miyazaki H, Tsuji M, Hanada-Dateki T, Araki T, Tomokiyo K, Hamamoto T, Ogata Y. Combining FVIIa and FX into a mixture which imparts a unique thrombin generation potential to hemophilic plasma: an in vitro assessment of FVIIa/FX mixture as an alternative bypassing agent. *Thromb Res* 2010;125:457-63.
- [41] Lindley CM, Sawyer WT, Macik BG, Lusher J, Harrison JF, Baird-Cox K, Birchlazer S, Roberts HR. Pharmacokinetics and pharmacodynamics of recombinant factor VIIa. *Clin Pharmacol Ther* 1994;55:638-48.
- [42] Mori K, Sakai H, Nakano N, Suzuki S, Sugai K, Hisa S, Goto Y. Congenital factor X deficiency in Japan. *Tohoku J Exp Med* 1981;133:1-19.

- [43] Ostermann H, Haertel S, Knaub S, Kalina U, Jung K, Pabinger I. Pharmacokinetics of Beriplex PN prothrombin complex concentrate in healthy volunteers. *Thromb Haemost* 2007;98:790-7.
- [44] Tomokiyo K, Nakatomi Y, Araki T, Teshima K, Nakano H, Nakagaki T, Miyamoto S, Funatsu A, Iwanaga S. A novel therapeutic approach combining human plasma-derived factors VIIa and X for haemophiliacs with inhibitors: evidence of a higher thrombin generation rate in vitro and more sustained haemostatic activity *in vivo* than obtained with factor VIIa alone. *Vox Sang* 2003;85:290-9.
- [45] Bjoern S, Thim L. Activation of coagulation factor VII to VIIa. *Research Disclosure* 1986;269:564-65.
- [46] Tomokiyo K, Yano H, Imamura I, Nakano Y, Nakagaki T, Ogata Y, Terano T, Miyamoto S, Funatsu A. Large-scale production and properties of human plasma-derived activated factor VII concentrate. *Vox Sang* 2003;84:54-64.
- [47] Downey C, Kazmi R, Toh CH. Early identification and prognostic implications in disseminated intravascular coagulation through transmittance waveform analysis. *Thromb Haemost* 1998;80:65-9.
- [48] Matsumoto T, Wada H, Nishioka Y, Nishio M, Abe Y, Nishioka J, Kamikura Y, Sase T, Kaneko T, Houdijk WP, Nobori T, Shiku H. Frequency of abnormal biphasic aPTT clot waveforms in patients with underlying disorders associated with disseminated intravascular coagulation. *Clin Appl Thromb Hemost*. 2006;12:185-92
- [49] Shima M. Understanding the hemostatic effects of recombinant factor VIIa by clot waveform analysis. *Semin Hematol*. 2004;41(Suppl 1):125-31.
- [50] Fukui H, Fujimura Y, Takahashi Y, Mikami S, Yoshioka A: Laboratory evidence of DIC under FEIBA treatment of a hemophilic patient with intracranial bleeding and high titer factor VIII inhibitor. *Thromb Res* 1981;22:177-184
- [51] Rodeghiero F, Castronovo S, Dini E: Disseminated intravascular coagulation after infusion of FEIBA (factor VIII inhibitor bypassing activity) in a patient with acquired hemophilia. *Thromb Haemost* 1982;48:339-40
- [52] Chavin SI, Siegel DM, Rocco TA, Olson JP: Acute myocardial infarction during treatment with an activated prothrombin complex concentrate in a patient with factor VIII deficiency and a factor VIII inhibitor. *Am J Med* 1988;85:245-9
- [53] Shirahata A, Fukutake K, Mimaya J, Takamatsu J, Shima M, Hanabusa H, Takedani H, Takashima Y, Matsushita T, Tawa A, Higasa S, Takata N, Sakai M, Kawakami K, Ohashi Y, Saito H. Clinical pharmacological study of a plasma-derived factor VIIa and factor X mixture (MC710) in haemophilia patients with inhibitors - Phase I trial. *Haemophilia* 2011 in press
- [54] Shirahata A, Kamiya T, Takamatsu J, Kojima T, Fukutake K, Arai M, Hanabusa H, Tagami H, Yoshioka A, Shima M, Naka H, Fujita S, Minamoto Y, Kamizono J, Saito H. Clinical trial to investigate the pharmacokinetics, pharmacodynamics, safety, and efficacy of recombinant factor VIIa in Japanese patients with hemophilia with inhibitors. *Int J Hematol* 2001;73:517-25.
- [55] Elg M, Carlsson S, Gustafsson D. Effect of activated prothrombin complex concentrate or recombinant factor VIIa on the bleeding time and thrombus formation during anticoagulation with a direct thrombin inhibitor. *Thromb Res* 2001;101:145-57.
- [56] Gringeri A, Fischer K, Karafoulidou A, Klamroth R, López-Fernández MF, Mancuso E; ON BEHALF OF THE EUROPEAN HAEMOPHILIA TREATMENT STANDARDISATION BOARD (EHTSB). Sequential combined bypassing therapy is safe and effective in the treatment of unresponsive bleeding in adults and children with hemophilia and inhibitors. *Haemophilia* 2011 in press

Hemophilia

Edited by Dr. Angelika Batorova

ISBN 978-953-51-0429-2

Hard cover, 130 pages

Publisher InTech

Published online 30, March, 2012

Published in print edition March, 2012

This book demonstrates the great efforts aimed at further improving the care of the hemophilia, which may bring further improvement in the quality of life of hemophilia persons and their families.

How to reference

In order to correctly reference this scholarly work, feel free to copy and paste the following:

Kazuhiko Tomokiyo, Yasushi Nakatomi, Takayoshi Hamamoto and Tomohiro Nakagaki (2012). Prospective Efficacy and Safety of a Novel Bypassing Agent, FVIIa/FX Mixture (MC710) for Hemophilia Patients with Inhibitors, Hemophilia, Dr. Angelika Batorova (Ed.), ISBN: 978-953-51-0429-2, InTech, Available from: <http://www.intechopen.com/books/hemophilia/prospective-efficacy-and-safety-of-a-novel-bypassing-agent-fvii-a-fx-mixture-mc710-for-hemophilia-pat>

INTECH
open science | open minds

InTech Europe

University Campus STeP Ri
Slavka Krautzeka 83/A
51000 Rijeka, Croatia
Phone: +385 (51) 770 447
Fax: +385 (51) 686 166
www.intechopen.com

InTech China

Unit 405, Office Block, Hotel Equatorial Shanghai
No.65, Yan An Road (West), Shanghai, 200040, China
中国上海市延安西路65号上海国际贵都大饭店办公楼405单元
Phone: +86-21-62489820
Fax: +86-21-62489821

© 2012 The Author(s). Licensee IntechOpen. This is an open access article distributed under the terms of the [Creative Commons Attribution 3.0 License](https://creativecommons.org/licenses/by/3.0/), which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited.

IntechOpen

IntechOpen