Microvascular flow model to study clot formation in real-time

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Objective: Microvascular occlusion and thrombosis is a process involving several components as coagulation factors, endothelial cells, hemodynamic forces and the vessel geometry and is further affected by the complement system. So far most experiments examining the course of vessel occlusion have been performed in either animal models or isolated in vitro systems, which are either difficult to interpret and to transfer to human physiology or only focus on certain aspects in a non-physiological environment. A recently developed so-called “microvasculature-on-a-chip” model (CHIP) combines the physiological environment of animal models with the reproducibility and easy-handling of in vitro systems.

Methods: This microvascular flow model features channels in the micrometer range coated by a confluent monolayer of endothelial cells which can be perfused with whole blood (WB) at a physiological flow rate. The transparent character of the device (poly-dimethyl-siloxane) allows for real-time observation of the blood flow, thrombus formation and subsequent vessel occlusion by confocal microscopy.

Results: Here we visualize the crosslinking actions of FXIII in clot formation and study the effect of the complement enzyme mannan-binding-lectin-associated serine protease-1 (MASP-1) on clot formation in whole blood. MASP-1 resembles thrombin in terms of its structural features and substrate specificity and has been shown to be involved in fibrin clot formation in a prothrombin-dependent manner.

Perfusion of a CHIP with freshly drawn recalcified WB in presence or absence of physiological concentrations of a recombinant form of MASP-1 confirmed that rMASP-1 is able to significantly enhance clot formation in WB.

We visualized the action of FXIII in WB by staining the crosslinks of forming fibrin clots with a specific antibody, by observing the incorporation of specific FXIII substrates and by the use of FXIII inhibitors. The model further allows to examine the dissolution of the clot in real-time.

Conclusion: Taken together, this microvascular flow model allows to study the effect of various enzymes on the process of vascular occlusion and thrombogenesis in a close-to-physiological environment in real-time.
rhADAMTS13 treatment improves ventricular remodeling and functionality after cardiac injury in mice

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**Objective:** Myocardial injury leads to detrimental cardiac remodeling, scar tissue formation and, eventually, congestive heart failure. Von Willebrand factor, released from the stimulated vessel wall, mediates leukocyte and platelet recruitment and thereby promotes coronary inflammation and micro-vessel thrombosis. The metalloproteinase ADAMTS13 decreases the size and activity of ultra-large VWF and its infusion produces beneficial results 24 hours after myocardial ischemia reperfusion injury (MIRI) in mice.

**Methods:** We evaluated the effect of recombinant human ADAMTS13 (rhADAMTS13) treatment on long-term cardiac remodeling and functionality in mice undergoing surgical models of MIRI and chronic left ventricular pressure overload. Both models produce a coronary inflammatory response and micro-vascular dysfunction. Mice were treated with either rhADAMTS13 or vehicle and assessed for myocardial inflammatory cell recruitment, ventricular function and dimensions at different post-surgical time points, and for cardiac fibrosis after 4 weeks.

**Results:** We observed significant preservation of cardiac function and decrease in fibrotic remodeling as a result of rhADAMTS13 treatment in both models of cardiac injury. Coronary vascular inflammation and inflammatory cell recruitment in pressure-overloaded ventricles was attenuated in rhADAMTS13 treated mice.

**Conclusion:** Here we show that rhADAMTS13 improves cardiac remodeling after injury by MIRI or left ventricular pressure overload in mice. Our study further supports the therapeutic potential of rhADAMTS13 for myocardial infarction patients in the future and, potentially, in other conditions characterized by inflammatory cardiac damage that results in fibrosis.
Activated protein C is a sensitive biomarker to monitor the activity level of the hemostatic system after activation by recombinant activated factor VII

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Objective: Evaluation of the activity level of the hemostatic system using current biomarkers is difficult due to their high interindividual variation, low specificity, or long persistence in circulation. Objective of our study was to monitor the activation status of coagulation by measuring plasma levels of free thrombin and activated protein C (APC) using a highly sensitive oligonucleotide-based enzyme capture assay (OECA) platform.

Methods: In order to induce a standardized and limited activation of the clotting cascade, recombinant, activated factor VII (rFVIIa) was injected i.v. into 10 healthy volunteers (6 females) at a dose of 15 µg/kg bodyweight. Plasma levels of FVIIa and of hemostasis-related biomarkers including free thrombin, APC, the prothrombin activation fragment F1+2, thrombin-antithrombin complex (TAT), plasmin-alpha2-antiplasmin complex (PAP), and D-dimer were determined at baseline and repeatedly during a 24 h lasting follow-up period.

Results: rFVIIa was well tolerated, and its elimination kinetics showed an expected course in all probands. While APC plasma levels lay below the lower limit of quantification at baseline in all subjects, a highly significant increase of APC (median; interquartile range) was observed after 10 min (0.16 (0.12-0.17) ng/mL, p = 0.003), 30 min (0.19 (0.15-0.20) ng/mL, p = 0.005), 60 min (0.16 (0.15-0.20 ng/mL, p = 4x10^-5), and 120 min (0.13 (0.12-0.18) ng/mL, p = 7x10^-4). F1+2 slightly increased from 0.13 (0.10-0.21) nmol/L at baseline to 0.16 (0.11-0.23) nmol/L after 60 min, 0.18 (0.13-0.20) nmol/L after 120 min, and 0.18 (0.13-0.20) nmol/L after 180 min, but these changes lost statistical significance after correction for multiple testing. No significant changes of plasma levels of free thrombin, TAT, PAP and D-dimer were observed.

Conclusion: Among the biomarkers evaluated in this study, APC appears to be best suited to estimate the activation status of the hemostatic system. Our results warrant further studies to evaluate its use in clinical situations of coagulation activation.
In vitro evaluation of aptamer-based reversible inhibition of anticoagulant activated protein C as a novel supportive hemostatic approach

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Objective: It has been shown that overwhelming formation of activated protein C (APC) may play an important role in the pathogenesis of trauma induced coagulopathy (TIC). In addition, APC has been recognized as a candidate target for the adjuvant treatment of hemophilia. Thus, drugs that inhibit the anticoagulant activity of APC might be useful for the treatment of acute or chronic bleeding complications. The objective of the present study was the in vitro evaluation of HS02-52G, an APC-binding DNA-aptamer, as a reversible inhibitor of APC.

Methods: The binding kinetics of HS02-52G were determined by Biolayer Interferometry (BLI). A series of single-stranded (ss) DNA molecules (AD series), complementary to different parts of HS02-52G, were designed in order to identify an effective HS02-52G-neutralizing antidote molecule. The specificity as well as the APC-inhibitory potential of HS02-52G and the performance of the AD-molecules were assessed by various in vitro coagulation models, based on, inter alia, plasma or whole blood aPTT assays and the thrombin generation assay (calibrated automated thrombogram [CAT]).

Results: Regarding the binding of HS02-52G to APC, BLI analysis revealed on- and off-rates of $1.118 \pm 0.013 \times 10^5$ M$^{-1}$ s$^{-1}$ and $1.234 \pm 0.033 \times 10^3$ s$^{-1}$, respectively, corresponding to a KD of 11 nM. In contrast, only negligible binding to the zymogen protein C was observed. Even when applied at concentrations of 1 µM, no aptamer-specific effects on global coagulation assays could be observed, proving the high target-specificity of HS02-52G. Accordingly, functional analysis revealed HS02-52G as a potent inhibitor of APC in plasma and whole blood with IC50 values <= 30 nM. Regarding the neutralization of the APC-inhibitory activity of HS02-52G, the complementary ssDNA-molecule AD22 was found to be an effective HS02-52G-antidote in plasma and whole blood (found IC50 values <= 200 nM).

Conclusion: HS02-52G has been introduced as a highly specific APC inhibitor. It could be demonstrated that the aptamer retains its high inhibitory activity in plasma and whole blood and that its functional activity within these matrices can be effectively reversed by the short antisense molecule AD22. In the first place, these features qualify the novel native aptamer-antidote pair as a save candidate treatment option for acute APC-related bleeding complications as, for instance, potentially observed in TIC.
Regulation of complement and contact system activation via C1 inhibitor potentiation and factor XIIa activity modulation by heparins, glycosaminoglycans and other sulfated glycans

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Objective: C1 inhibitor (C1-INH) is the only regulator of classical complement activation as well as the major regulator of the contact system. Its importance is demonstrated by hereditary angioedema (HAE), a severe disease with potentially life-threatening attacks due to deficiency or dysfunction of C1-INH. C1 INH replacement is the therapy of choice in HAE. In addition, C1-INH showed to have beneficial effects in other diseases characterized by inappropriate complement and contact system activation. Due to some limitations of its clinical application, there is a need for improving the efficacy of therapeutically applied C1-INH or to enhance the activity of endogenous C1-INH. Given the known potentiating effect of heparin on C1-INH, sulfated glycans (SG) may be such candidates.

The aim of this study was to characterize suitable lead structures by investigating structurally distinct SG for their C1-INH potentiation activity as well as their direct effects on the complement factor C1s and on factor FXIIa (FXIIa).

Methods: The effects of SG on the C1INH-mediated inhibition of C1s and FXIIa as well as on C1s and FXIIa activity were measured by chromogenic substrate assays. The > 40 structurally defined SG tested in this study included (1) heparins with molecular weights (MW) ranging from 3 to 15 kDa and fondaparinux, (2) further genuine and chemically modified glycosaminoglycans (GAGs) and danaparoid (3) two series of high-MW and low-MW semisynthetic β 1,3 glucan sulfates, and (4) various algae-derived SG, and (5) two series of successively depolymerized fucoidans.

Results: The SG turned out to potentiate the C1s inhibition by C1-INH without any direct influence on C1s. Their potentiating activity proved to depend on their degree of sulfation, molecular mass as well as glycan structure. In contrast, the SG had no effect on the FXIIa inhibition by C1-INH, but structure-dependently modulated the activity of FXIIa. Among the tested SG, β-1,3-glucan sulfates with MW <= 10 000 were identified as most promising lead candidates for the development of a glycan-based C1-INH amplifier.

Conclusion: In conclusion, the obtained information on structural characteristics of SG favouring C1-INH potentiation represent an useful elementary basis for the development of compounds improving the potency of C1-INH in diseases and clinical situations characterized by inappropriate activation of complement and contact system.
Interim results from a dose escalating study of AMT-060 (AAV5-hFIX) gene transfer in adult patients with severe haemophilia B

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Objective: Gene transfer for haemophilia offers the potential to shift the disease severity from severe to mild with a single treatment. AMT-060 consists of an AAV5 vector containing a liver-specific promoter and codon-optimized wildtype hFIX gene. Here, we investigate the safety and efficacy of AMT-060 in adults with severe haemophilia B.

Methods: This is a Phase 1/2, multi-national, multi-center, open-label, dose-escalating study. Patients had to have FIX ≤2% of normal and require either prophylactic exogenous FIX, or on-demand exogenous FIX with either ≥4 bleeds/year or haemophilic arthropathy. Patients received AMT-060 at either 5x10^12 gc/kg (n=5) or 2x10^13 gc/kg (n=5) via a single intravenous infusion over 30 minutes. Assessments include endogenous FIX activity, measured >10 days after the most recent administration of exogenous FIX; reduction of exogenous FIX use; annualized spontaneous bleeding rates; treatment related adverse events; and immunological assessments.

Results: There were no screen failures for pre-existing anti-AAV5 antibodies. Ten patients enrolled (33-72 years of age) and received AMT-060; 9 were on FIX prophylaxis and 1 in the higher-dose cohort used on-demand FIX therapy. At the time of submission, mean endogenous FIX activity after cessation of prophylaxis in the low-dose cohort was 5.4% (95% CI 5.0-5.8%, range 3.1-6.7%; n=4), and stable during 39 weeks of follow-up. Four of 5 patients in the low-dose cohort were able to stop FIX prophylaxis, reducing their annualized total FIX usage by a mean of 82%. One patient in the low-dose cohort had a mild, asymptomatic elevation of ALT at week 10 post-AMT-060 that resolved with a 7 weeks course of tapering prednisolone. No change in FIX activity, T-cell response, or other immunogenicity/inflammatory abnormalities were seen during the ALT elevation. Results will be updated up to 52 weeks for the low-dose cohort. Initial results up to 26 weeks will be presented for the higher-dose cohort.

Conclusion: Follow up of patients with severe haemophilia B who received AMT-060 is ongoing. A single infusion of AMT-060 was generally well-tolerated. FIX activity increased to levels sufficient to provide endogenous prophylaxis in 4/5 patients in the low-dose cohort, relieving them from the need for exogenous FIX prophylaxis and resulting in marked decrease of FIX usage.