Application of reversible inhibitors for hemocompatible release systems

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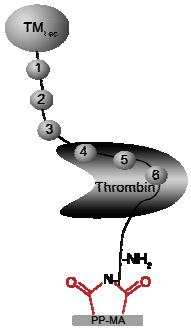
Background and Objectives

- **Clinical problem: Activation of blood coagulation in contact with biomedical devices**
- ⇨ **Risk of thromboembolic organ infarcts**
- ⇨ **Increased risk of bleeding upon prophylactic systemic anticoagulation**

Thrombomodulin as ideal



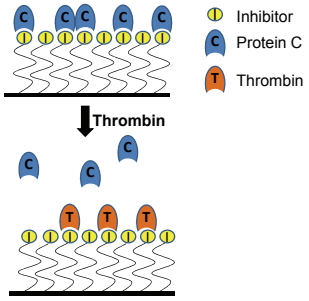
Leading anticoagulant and antiinflammatory molecule in the endothelial cell membrane.

- Inhibits coagulant thrombin activity
- Activates anticoagulant protein C pathway
- Antiinflammatory properties

Disadvantages of immobilization
- Low stability at sterilization
- Low shelf stability
- In vivo degradation
- High expenses

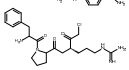
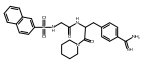
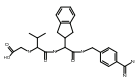
Concept to simulate thrombomodulin function by use of small inhibitors

- Thrombin and activated Protein C (APC) are structurally related serine proteases
- Competitive inhibitors can show cross-specifity
- Thermodynamics favors inhibitor binding to an enzyme, depending on concentration or affinity
- ⇨ **Concept for a thrombin-responsive release system of APC:**
- Immobilized inhibitor
- Pre-loading with APC (Xigris, Drotrecogin alfa)
- **Blood contact**:
- Inhibitor-immobilized form protects APC from inactivation by serpins
- Thrombin from coagulation processes replaces APC at the inhibitor

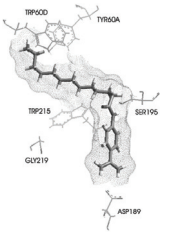


Concept

Inhibitor-Molecules
Inhibitor constants were determined using various concentrations (50-300 µM) of the chromogenic substrates S-2238 (Thrombin) or S-2366 (APC). The inhibition of the turnover by inhibitor molecules was determined photometrically. Inhibitor constants were calculated in the Dixon plot.

		Inhibitor constants	
		Thrombin [µM]	APC [µM]
p-amino benzamidine		26 288 ± 7 661	3 481 ± 2 204
4-aminomethyl benzamidine		32 052 ± 8 084	14 856 ± 3 509
TI-2[1]		2 397 ± 1 160	4 580 ± 2 101
PPACK		(irreversible)	(irreversible)
NAPAP		1.1 ± 0.4	15.4 ± 4.5
C-26[2]		5 622 ± 3 775	3.0 ± 0.4

Inhibitor constants
Small inhibitors have very low affinity to the enzymes .
More complex inhibitors have higher affinity and selectivity for specific proteases.
NAPAP: good selectivity for thrombin
C26: APC inhibitor

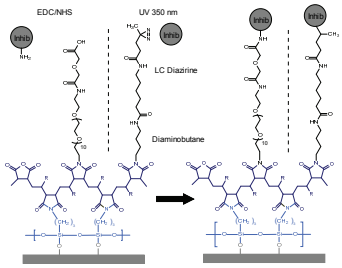


Thrombin inhibitor
Sketch of Inhibitor TI-2 in reactive center of Thrombin. Interaction of amidine with Asp-189

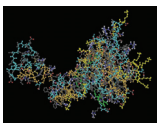
[1] Gouzy M.-F. *et al*. **In vitro blood compatibility of polymeric biomaterials through covalent immobilization of an amidine derivative.** *Biomaterials* ***25:*** 3493-3501 (2004)
[2] De Nanteuil G. *et al*. **Low molecular weight activated protein C inhibitors as a potential treatment for hemophilic disorders.** J Med Chem **49**: 5047-5050 (2006)

Results

Immobilization
Polypropylene maleic anhydride copolyers served as substrates.
- Amine bearing inhibitors : Immobilization via a PEG12 –spacer using EDC/NHS chemistry
- Amine-free inhibitors : Immobilization via photocrosslinker LC-Diazirine



Grafting density
Estimation of required inhibitor density for APC monolayer loading:
APC footprint: 5.7 × 8.9 nm². Theoretical monolayer concentration 3.27 pmol/cm².



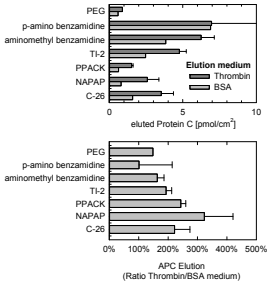
Inhibitors are grafted at higher density than necessary for APC monolayer formation

Determined density
Hydrolysis of immobilized inhibitors and amino acid quantification by HPLC

Substance	Surface concentration [nmol/cm²]
PPACK	0.36
NAPAP	1.37
C-26	0.56

APC release
Method:
- Loading of immobilized inhibitors with ^{125}I conjugated APC.
- Elution with Tris-BSA buffer with or without thrombin

Result:
- Thrombin elutes more APC than BSA alone
- Low affinity inhibitors show high non-specific adsorption of APC.
- Selective thrombin inhibitor NAPAP appears best for selective release of APC by thrombin.



Conclusions

- APC loaded immobilized reversible inhibitors are suggested as a thrombin responsive release system
- Their function simulates the effect of the vessel wall protein APC for short term
- Complex inhibitors allow more specific binding and release of APC
- Up to now no benefit in whole blood seen. Optimization of immobilization conditions/substrate properties required

Acknowledgement
This study is funded by Deutsche Forschungsgemeinschaft DFG.

The inhibitor C-26 was a generous gift of Mr. De Nanteuil

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Aim: Blood clotting is an ongoing challenge of biomedical devices in contact with blood. Frequently heparin or proteins of the blood vessel wall are immobilized to a device surface in order to achieve hemocompatibility. Here, an alternative approach is presented, which mimics the function of the vessel wall protein thrombomodulin: inactivation of the coagulant thrombin activity and release of the anticoagulant Activated Protein C (APC).

Concept: The anticoagulant serine protease APC structurally related to the coagulation factors, such as thrombin. Competitive inhibitor molecules targeting the reactive center of the enzymes show cross specifity. According to thermodynamics, the inhibitor will preferentially bind to an enzyme, where it has higher affinity or which is present at higher concentration. This can be used to build up an on demand APC release system using an immobilized reversible inhibitor preloaded with APC. Upon coagulation activation, the excessively formed thrombin replaces APC from the inhibitor. The anticoagulant APC is released into the blood fluid and interrupts the coagulation cascade, while the coagulation factor thrombin binds to the inhibitor and gets inactivated.

Method: Benzamidine based thrombin inhibitors were surface-immobilized via PEG spacers. They were loaded with radio-conjugated APC and the release was tested with thrombin or with non-competitive albumin.

Result: The various inhibitors showed different affinities and selectivities for Protein C. All surfaces released more APC in presence of thrombin compared to the albumin control. Thus, a release system for the anticoagulant Activated Protein C has been built up, which is responsive to an enhanced coagulation situation and has direct thrombin inhibiting properties. The system needs further optimization in blood plasma and in whole blood incubation assays.