Optimization of the antiidiotypic DARPin selection needed to develop a broadly neutralizing drug for acquired thrombotic thrombocytopenic purpura (aTTP)

1. Abstract

Thrombotic thrombocytopenic purpura (TTP) is a rare and life-threatening coagulation disorder first described in 1924. TTP is classified as acquired (aTTP) or hereditary, both defined by a severe ADAMTS13 deficiency leading to thrombocytopenia, microvascular thrombosis, microangiopathic hemolytic anemia and organ dysfunction. Autoimmune aTTP is caused by inhibitory anti-ADAMTS13-antibodies. Todays standard treatment is plasma exchange (PEX) combined with corticosteroids, untreated the mortality rate is 90%. Surviving patients have a 50% risk of relapse, in this case second line treatments like Rituximab or splenectomy are commonly applied. To treat relapsing patients, a new therapy approach aiming directly at the inhibitory antibodies would be ideal. From two relapsing female patients donating their spleens, anti-ADAMTS13 antibodies were extracted and used to select anti-idiotypic small binding molecules, Designed Ankyrin Repeat Proteins (DARPins) from a molecular library. DARPins have already been shown to bind and neutralize anti-ADAMTS13-antibodies in aTTP plasma samples, so the main topic of this thesis is the evaluation of the neutralization potential of single and pooled DARPins using the FRETS-VWF73 (fluorescence resonance energy transfer substrate - von Willebrand factor 73) assay. The main finding is, that different DARPins must be combined to achieve a neutralization of the inhibitory autoantibodies of aTTP patients.

2.1 aTTP

Aetiologically, aTTP is an autoimmune disease with autoantibodies either inhibiting ADAMTS13 activity or accelerating its clearance. This results in a severely reduced processing of von Willebrand factor (VWF), a large, multimeric glycoprotein present in human plasma. If VWF is not continuously broken down into smaller fragments, it promotes spontaneous platelet aggregation and clot formation [1].

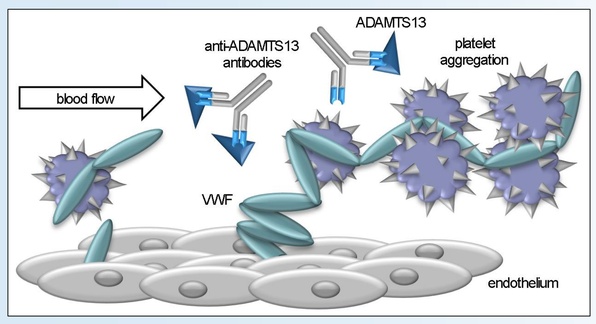

Figure 1
Pathophysiology of aTTP: Autoantibodies binding ADAMTS13 inhibit the processing of VWF. Especially large multimers can bind great numbers of platelets and cause thrombosis.

2.2 DARPins

Designed Ankyrin Repeat Proteins (DARPins) are a novel class of binding molecules, originating from the natural ankyrin proteins found in the human genome. The DARPin technology already has been shown to be effective as a treatment in studies for a different disease.
DARPins are built from artificially designed, tightly joined ankyrin repeat modules, consisting of constant (framework) and variable amino acid residues (individual target binding site) [2].
The DARPins used are divided upon different motifs (1, 3 and 4), given by the CDR3-sequence of the antibody selecting them during ribosome display.

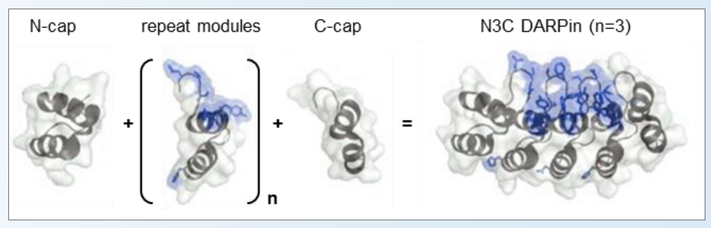

Figure 2
General DARPin structure: A hydrophilic N- and C-cap enclose the respective number of repeat modules, here 3, thus forming a N3C DARPin. The blue regions represent the variable amino acids forming the target binding site.

3. Goals and main questions

1. Contribution of individual DARPins in neutralizing anti-ADAMTS13 autoantibodies in plasma of patients suffering from a first episode of acquired TTP?
2. Improvement using a combination of individual DARPins within one motif and/or from multiple motifs?
3. Is the neutralization potential different in aTTP patients with a relapsing disease course?

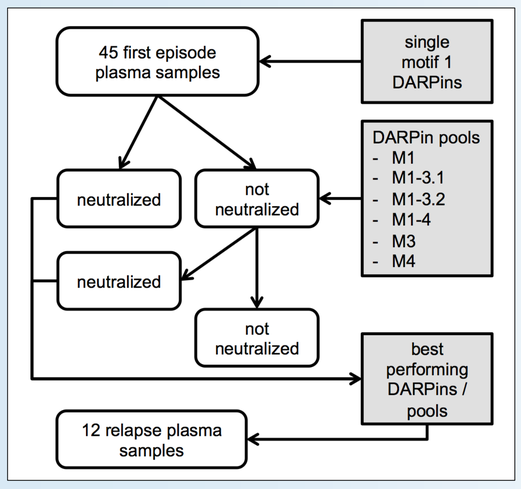

Figure 3
Workflow:
1) Neutalization potential of single DARPins
2) Neutalization potential of 6 different pools
3) Most potent DARPins/pools are tested with relapse samples

4. Materials&Methods

Patient cohort (n=56): The patient sample selection criteria were a confirmed aTTP diagnosis, a sample drawn before PEX was initiated with an ADAMTS13 activity below 5% and a inhibitor titer greater than 1 Bethesda Units per ml (BU/ml). All samples (n=57) were diluted to achieve titers between 1 and 2 BU/ml (except the relapse samples (n=12)).

To measure the proteolytic activity of ADAMTS13 in plasma samples, the FRETS-VWF73 method was performed. This kinetic assay relies on fluorescence resonance energy transfer (FRET) and a chemically synthesized fluorogenic peptide, FRETS-VWF73 (Peptide Institute Inc.), as the substrate for ADAMTS13. If FRETS-VWF73 is cleaved, fluorescence is emitted, which is measured over time [3].
To measure how DARPins affect the inhibitor titer, they are added to the aTTP plasma samples and incubated 1h. Standard Human Plasma (Siemens) then is added in equal amounts (=50% ADAMTS13 activity) and incubated another hour. After incubation, the residual ADAMTS13 activity is measured, so the inhibitor titer can be calculated from the activity decrease.
The neutralization potential is the difference between titer measured with and without DARPins added.

Motif-1 Pools				Motif 3 Pool	Motif 4 Pool
M1	M1-3.1	M1-3.2	M1-4	M3	M4
N2C-9	N2C-9	N3C-74	N2C-9	N2C-7	N2C-87
N2C-19	N2C-19	N2C-90	N2C-19	N2C-34	N2C-95
N2C-47	N2C-47	N3C-158	N3C-74	N2C-36	N2C-114
N3C-74			N2C-90	N2C-52	N2C-143
N2C-90				N2C-55	N2C-156
N3C-158				N2C-83	N2C-166
				N2C-110	N2C-167
				N2C-124	
				N2C-142	

Table 1
The different pools with the DARPins they consist of.

5. Results

1. No single motif-1 DARPin lowered the inhibitor titer under the pathological threshold of 0.4 BU/ml.
2. The pools containing motif-1 DARPins (M1, M1-3.1, M1-3.2, M1-4) showed a significantly superior neutralization potential compared to the motif-3 and -4 pools (as seen on figure 4). The average inhibitor titer (1.74 BU/ml) was reduced by 60% with M1, 45% by M1-3.1, 31% by M1-3.2, 47% by M1-4 (on average). 7/22 inhibitor titers were neutralized (lowered below the threshold) by M1.
3. 12 samples from aTTP patients with a relapsing disease course were tested with the DARPin pools M1 and M1-4. In 9/12 samples (M1) respectively 7/12 (M1-4) the ADAMTS13 activity was restored by decreasing inhibitor titers below the pathological threshold (figure 5).

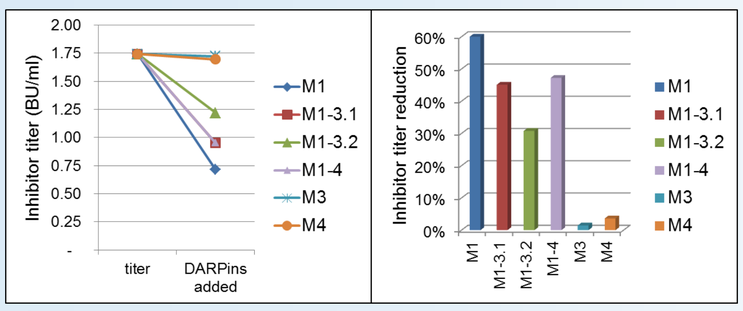

Figure 4
Left panel: Average inhibitor titer reduction of the different pools
Right panel: Percentage of titer reduction

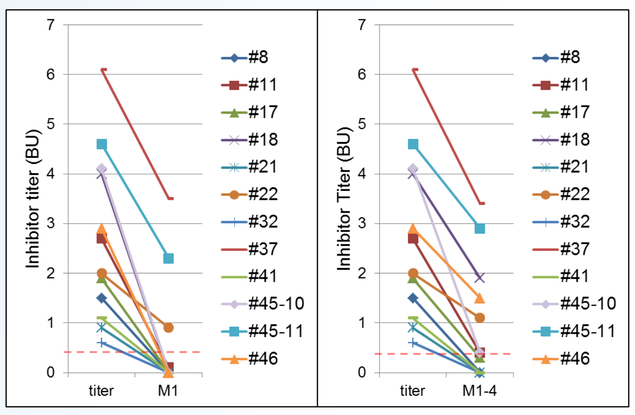

Figure 5
Titer reduction for each relapsing patient (n=12) for DARPins pool M1 (left panel) and M1-4 (right panel)

6. Discussion

1. The low neutralization potential of single DARPins might be due to the known polyclonal nature of the immune response towards ADAMTS13 [4]. In order to achieve an universal neutralization, a combination of different DARPins is needed.
2. Pooled DARPins of motif-1 showed a greater neutralization potential than the pooled DARPins of motif-3 and -4, suggesting that their epitope has a greater resemblance of that of ADAMTS13. The DARPins N2C-9 and N2C-19 (motif-1) seem crucial.
3. The observed higher neutralization potential in plasma of patients suffering from a relapsing course compared to acute first episode might be due the fact that the antibodies used to select the DARPins originate from 2 relapsing aTTP patients.

Outlook:
To confirm the data, tests with more samples, respectively a larger patient cohort, will be performed.
DARPins, being a very versatile scaffold, could easily be modified as linking to a toxin for a direct targeted killing of autoantibody producing B-cells.

Bibliography
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Figures & Tables
Figure 2: Stumpp, M.T., Binz, H.K. and Amstutz, P. (2008) DARPins: a new generation of protein therapeutics | Drug Discov Today Vol 13(15-16): p. 696, Elsevier (adapted)
Others: Kocher, O. (2015), Bern, own figures/tables

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