The RNA aptamer, Apta-1 targeting evolutionarily conserved motif on thrombin cures endotoxemia and reduces thrombosis in animal models by inhibiting aggregation and secretion of platelets.

Authors, L. JEDLINA [1], O.CHUTNA OLIN [1] G. PARAMEL [2], K. FRANZEN [2] M. LINDSTAM [1], M. GRENEGÅRD [2]
1 Aptahem, Malmö, Sweden
2 Cardiovascular Research Centre, School of Medical Sciences, Örebro University, Örebro, Sweden

ISTH 2023 JUNE 24-28 CONGRESS #ISTH2023 ISTH2023.ORG montréal

APTAHEM

INTRODUCTION

Systemic inflammation and thrombosis pose significant health challenges in various diseases, including sepsis. Current therapeutic approaches often encounter limitations, highlighting the urgent need for innovative therapeutic solutions that can effectively target inflammatory and thrombotic events. In this context, the RNA aptamer Apta-1 emerges as a promising candidate, specifically targeting an evolutionarily conserved motif on thrombin (exosite II). Thrombin activates platelets and other cell types via proteolytic cleavage of protease-activated receptors (PARs)[1], contributing to thrombosis and inflammation [2].
We present here the potential of Apta-1 in curing endotoxemia and reducing thrombosis in animal models by inhibiting platelet aggregation.

AIM

Apta-1 is a 90-mer synthetic RNA aptamer designed for therapeutic intervention in sepsis. This research aimed to evaluate the therapeutic potential of Apta-1 in thrombosis and sepsis animal models and to elucidate the underlying mechanism of action.

METHODS

- The NanoTemper microscale thermophoresis method was used to detect Apta-1 binding to the fluorescently labelled thrombin and to calculate a dissociation constant (Kd) **(Figure 1)**.
- To study the effect of Apta-1 on thrombin function and thrombin generation, several *in vitro* assays were employed:
  - The total amount of thrombin generated in human blood treated with either Apta-1 in concentrations ranging from 0.8 µM to 12.8 µM or with heparin sodium (0.7 IU/mL) was assessed by a thrombin generation assay in citrated plasma **(Figure 2A)**.
  - To determine if Apta-1 affected thrombin`s ability to cleave fibrinogen, human citrated plasma was incubated at 37°C for 1 min with thrombin (0.5 U/ml), thrombin premixed with Apta-1 (6 µM), or E. coli total RNA (at a dose corresponding to 6 µM Apta-1). After incubation, the enzymatic activity of thrombin was neutralized by adding 2.5 U/ml hirudin. The amount of released fibrinopeptide A in human plasma was measured by a commercial ELISA (ABIN 6955794) **(Figure 2B)**.
  - PAR-1 cleavage by thrombin: Isolated platelets from human blood were stimulated with either Apta-1 (3 µM) or equivalent amount of *E.coli* RNA in presence or absence of thrombin (0.5 U/mL). Control samples were treated with saline. Total proteins were extracted and subjected to Western-blot using PAR-1 cleaved-Ser42 antibody (abx015616; Abbexa) **(Figure 3)**.
- To assess the effect of Apta-1 on platelet activation, human whole blood was stimulated with thrombin (0.2 U/mL) in presence of Apta-1 (1 µM - 6 µM) or saline (control). Platelet aggregation was assessed by Multiplate® (Roche Diagnostics) during 6 min after stimulation with thrombin **(Figure 4A)**. For measurement of ATP secretion, citrated whole blood was diluted 1:1 with saline. ATP secretion from platelets was determined by performing a luciferin/luciferase assay according to the manufacturer's protocol (Chrono-Log Cooperation) **(Figure 4B)**.
- To explore the effects of Apta-1 on thrombosis *in vivo*, two rat models were used: ferric chloride carotid artery thrombosis model and arteriovenous shunt model for thrombosis. Thrombus weight was measured in both models **(Figure 5A and 5B)**.
- In order to mimic the human Systemic Inflammatory Response Syndrome (SIRS) we used mice **(Figure 6)** and non-human primates **(Figure 7)** *in vivo* models of endotoxemia where animals were challenged with endotoxin lipopolysaccharides (LPS), in presence or absence of Apta-1 treatment. Survival was monitored for 72h **(Figure 6)**. Blood samples were collected 3 or 24h post LPS challenge and complement fragments were quantified by flow cytometry using human anaphylatoxin cytometric bead array kit **(Figure 7)**.

RESULTS

Figure 1. Apta-1 binds to thrombin with a dissociation constant of approximately 30 nM.

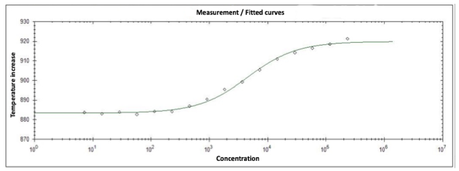
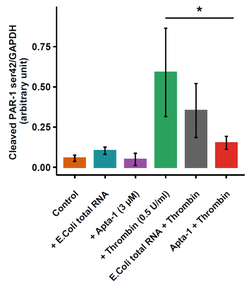


Figure 3. Apta-1 inhibits thrombin-induced PAR-1 cleavage on platelets.



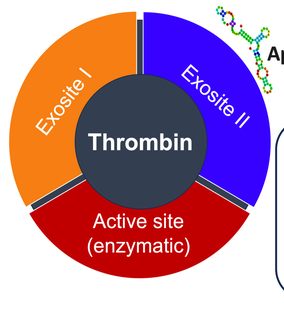
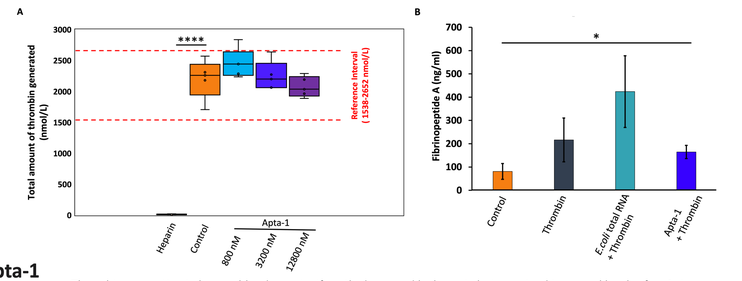


Figure 2. Apta-1 does not inhibit thrombin generation (A) or function of exosite I on thrombin (B).



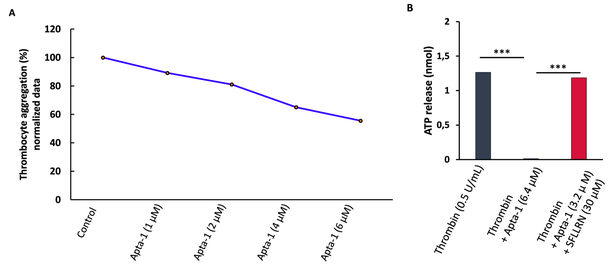
Thrombin generation in human blood was significantly decreased by heparin but remained in normal levels after Apta-1 treatment (A). Thrombin`s capacity to cleave fibrinogen, as measured by the amount of released fibrinopeptide A in human plasma, was not affected by Apta-1 (B).

Exosite I - Thrombin's exosite I interacts with molecules such as fibrinogen and thrombomodulin. It's important for cutting fibrinogen and starting the formation of blood clots. **Apta-1 does not affect this part of thrombin.**

Exosite II - Thrombin's exosite II interacts with platelet receptors, such as glycoprotein Ib and protease-activated receptors (PARs). This interaction triggers platelet activation and aggregation, leading to the formation of a stable blood clot. **Apta-1 inhibits these processes by binding to exosite II.**

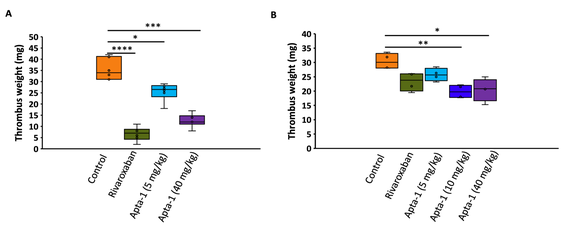
Active Site - Thrombin's catalytic site. It cleaves specific peptide bonds in its substrate molecules, including fibrinogen, activating various factors involved in blood coagulation and generating additional thrombin molecules through a positive feedback loop. **Apta-1 does not affect this part of thrombin.**

Figure 4. Apta-1 inhibits platelet aggregation (A) and secretion (B) induced by thrombin.



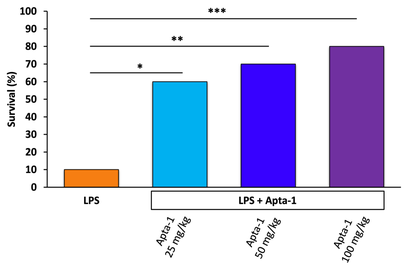
Platelet aggregation following stimulation with thrombin was inhibited by Apta-1 in human blood in a dose-dependent manner. Apta-1 at a dose of 6 µM reduced platelet aggregation by 44,4% (A). The release of ATP from platelet-dense granules was nearly abolished in the presence of Apta-1 (B). When the PAR-1 agonist SFLLRN hexapeptide was added, ATP secretion returned to normal levels, indicating that PAR-1 receptors remain fully functional.

Figure 5. Apta-1 inhibits thrombus weight in both carotid artery thrombosis ferric chloride rat model (A) and in arteriovenous shunt rat model of thrombosis (B).



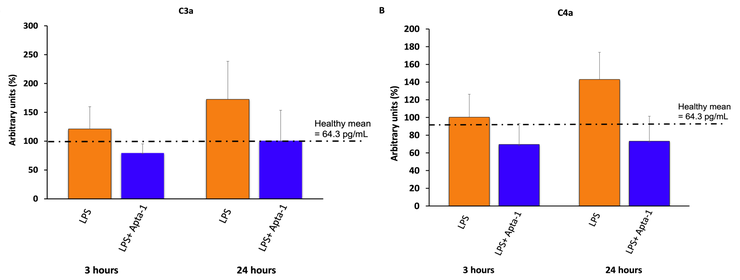
Apta-1 at doses of 5 and 40 mg/kg significantly diminished thrombus formation compared to the control group in a rat ferric chloride model (A). In an arteriovenous shunt rat model Apta-1 significantly decreased thrombus formation in doses of 10 and 40 mg/kg (B).

Figure 6. Apta-1 dramatically improves survival of endotoxemic mice.



Mice challenged with LPS endotoxin were monitored for 72 hours and survival was recorded. Survival in untreated group (n=10) was only 10%. Survival in groups treated with Apta-1 at a dose of 25 mg/kg, 50 mg/kg and 100 mg/kg (n=10 in each group) was dose dependent and ranged from 60 to 80%.

Figure 7. Apta-1 prevents excessive activation of complement cascade in non-human primates (NHP) model of endotoxemia.



The amount of complement fragments C3a (A) and C4a (B) in NHP treated with Apta-1 does not exceed the amounts observed in healthy animals during first 24 hours after LPS administration. Apta-1 prevents over-activation of complement cascade at early stages and thus exhibits anti-inflammatory effect in NHP endotoxemia animal model.

SUMMARY

Apta-1, a novel **RNA aptamer**:

- Binds to thrombin
- Significantly inhibits thrombin`s ability to activate platelets
- Simultaneously, it does not inhibit thrombin generation nor thrombin`s enzymatic activity of cleaving fibrinogen to fibrin, thus distinguishing itself from anticoagulants such as heparin.
- Acts as an antithrombotic *in vivo*
- Prevents hyperinflammatory response in an LPS-induced endotoxemia model
- Dramatically improves survival in an animal model of endotoxemia.

CONCLUSIONS

Apta-1, an RNA aptamer targeting an evolutionarily conserved motif on thrombin, exosite II, showcases its compelling potential as a drug candidate for acute inflammatory and thrombotic disorders. With its antithrombotic and anti-inflammatory properties, Apta-1 effectively curbs endotoxemia and mitigates thrombosis in animal models by inhibiting platelet aggregation and secretion, without the associated risk of bleeding.

ACKNOWLEDGEMENTS

The authors would like to acknowledge:
•Karin Strandberg, MD and My Chi Lam at Department of Clinical Chemistry and Pharmacology, Division of Laboratory Medicine, Coagulation Laboratory, University and Regional Laboratories Region Skåne in Malmö, Sweden for support with Thrombin generation assay and Multiplate aggregation assay in human blood samples.
•Anders Bylock, MD for advice on dosing in *in vivo* mice study.

REFERENCES

1 Foley JH and Conway EM Cross talk pathways between coagulation and inflammation. *Circulation Research 2016; 118:1392–1408*
2 Morrell CN et al. Emerging roles for platelets as immune and inflammatory cells. *Blood 2014; 18: 2759–2767*

Contact information:
For more information contact Luiza Jedlina, PhD
CSO at Aptahem AB
lj@aptahem.com
www.aptahem.com