SHORT THESIS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY (Ph.D.)

COLLAGEN AND THROMBIN, TWO OF THE CRUCIAL COMPONENTS OF THROMBUS FORMATION

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The Examination takes place at the library of the Department of Pediatrics, Medical and Health Science Center, University of Debrecen,
11 am, 12th December 2012

Head of the **Defense Committee**:	Dr. Gyorgy Balla MD, Member of the Hungarian Academy of Sciences
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Introduction

Hemostasis is the process that maintains the integrity of a closed, high-pressure circulatory system after vascular damage. When the vessel wall is breached or the endothelium is disrupted, collagen and tissue factor become exposed to the flowing blood, thereby initiating formation of a thrombus. Exposed collagen triggers the accumulation and activation of platelets, which rapidly adhere to collagen and various glycoprotein, activated and aggregate, a process that amplified by the local generation of thrombin. Whereas exposed tissue factor initiates the generation of thrombin. These events occur concomitantly, and under normal conditions, regulatory mechanisms contain thrombus formation temporally and spatially.

When pathologic processes overwhelm the regulatory mechanisms of hemostasis, excessive quantities of thrombin are formed, initiating thrombosis. Thrombosis is a critical event in the arterial diseases associated with myocardial infarction and stroke, and venous thromboembolic disorders that account for considerable morbidity and mortality. This is why it's important to understand the mechanism of thrombus formation and its regulation by antithrombotic agents.
During the period of my PhD study I got knowledge on the field of haemostasis and thrombosis and focused my research on two major components involved in thrombus formation: the collagen, and the thrombin.
Collagen is one of the most thrombotic surfaces on which thrombus is growing when endothelium is damaged. Several collagen types occur in the vessel wall, of which fibrillar collagen types I and III are considered to be the most important in supporting platelet adhesion to the damaged vasculature . Both types are also highly concentrated in fibrous atherosclerotic plaque. Three polypeptide chains form the fundamental structure of the collagen molecule, which is characterized by the presence of one or more triple-helical monomers. Within, these monomers, the three á-chains, wind around one another in a characteristic left-handed triple helix. The triple-helical monomers self-associate to form typical highly ordered collagen fibers. This conformation of collagen is of crucial importance, since the three-dimensional structure is needed for recognition of collagen by its ligands .One of the ligands is the von Willebrand factor (VWF). At arterial shear rates, collagen binds VWF from circulating blood and then platelets are captured and tethered on VWF immobilized by collagen, through the platelet surface receptor GPIb-IX-V, initiating thrombus formation. This is followed by platelet activation mediated by

the binding of ligands like GPVI to collagen, leading to inside-out stimulation of integrins $\alpha_2\beta_1$ and $\alpha_{IIb}\beta_3$ and maintaining platelet thrombus formation. The adhesive activity of the VWF is crucial in this process. Binding of VWF to collagen can be measured quantitatively and it is recommended as a method for determining VWF adhesive activity (VWF:CB). The assay is based on the measurement of VWF molecules bound to collagen by a VWF-specific antibody, similarly to the procedure for an enzyme-linked immunosorbent assay for VWF:Ag measurement. Several VWF:CB assays have been developed, and collagen binding activity of VWF is measured routinely when differential diagnosis of von Willebrand disease is needed. Differences between ways of preparing collagen solutions and differences between ways of using them to coat microplates may influence the result significantly.

Furthermore collagen is also a frequently used matrix in platelet adhesion assays. Platelet adhesion assay is used to study platelet physiology and one of the tests of platelet function in the clinical practice. This assay closely reflect the physiological milieu of platelets, including flow, red blood cells, and other blood components, regulating the interaction between subendothelial proteins and plasma proteins with platelet receptors resultant in adhesion and aggregation (thrombus formation). Measurement of platelet adhesion in whole blood samples under flow condition may be achieved by few technologies, for example in a collagen coated capillary, a parallel plate chamber, and cone and plate device. In this study I used the cone and plate apparatus which induces a laminar flow with uniform shear stress over the entire plate surface covered by the rotating cone.

In a survey of articles reporting the use of collagen-coated surfaces for VWF binding or for platelet adhesion, we noted the collagen type and its source, the concentration of collagen, the type of solvents used for coating the surface, the pH, and the coating conditions vary varied. These results implicate that studies on collagen-VWF binding and platelet thrombus formation need a uniform collagen matrix. Whether possible structural changes during the coating process enable better binding of the collagen to the surface and/or VWF binding to the collagen has not been studied systematically. To be able to make a uniform matrix, **one of the aims of my PhD work was to test the matrices of human collagen type-I and type-III prepared under different conditions in order to improve their ability to bind VWF and to support platelet adhesion.**

The serine protease thrombin plays a pivotal role in hemostasis; besides converting fibrinogen into fibrin clot, it activates factor V, FVIII and FXI, and triggers platelet activation through interaction with protease-activated receptors (PARs) 1 and 4 and glycoprotein Ib□ . Thrombin also exerts anticoagulant effect by activating protein C and thrombin activatable fibrinolysis inhibitor. Because of its central role in thrombus formation, thrombin is a major target for anticoagulation therapy. I studied a new class of thrombin inhibitors, the “thrombin aptamer”.
20 years ago Ellington et al found oligonucleotide molecules that bind specific ligands. Those molecules named 'aptamer' literally mean "to fit"(aptus) in latin.

Aptamers are single stranded nucleic acids that directly bind to biomedical relevant protein by folding into a specific three-dimensional structure with affinities and specifities that are comparable to antibodies. Aptamers are selected through iterative in vitor selection techniques known as SELEX (Systematic Evolution of Ligands by Exponential enrichment). Aptamers are considered to be an alternative to antibodies because their unique merits, such as thermal stability, low cost and immunogenicity, easily modified, and can be generated against variety target such as ions or small molecules. Aptamers have a short half-life, due to nuclease degradation, and can be rapidly cleaned from the blood stream by kidneys and can be antagoninized via its inhibition using anti-sense sequences. Selection *in vitro* allows the use of aptamers containing chemically modified nucleotides. Modifications are introduced to attain the following:

1. Optimize their pharmacokinetic, pharmacodynamic profiles and to promote their safety
2. Provide more contacts between aptamers and their targets, which should stimulate obtaining of aptamers with higher affinity and specificity towards their targets.
3. Make them nuclease-resistant, which is of great importance when applying aptamers for diagnosis and therapy.
4. Introduction of fluorescent groups into an aptamer which is used for analysis of its binding to the protein target.

Bock et al. selected aptamers with a consensus sequence of 15 nucleotides that dramatically inhibited the ability of thrombin to clot fibrinogen. The consensus 15-mer oligonucleotide, [d(GGTTGGTGTGGTTGG)] (C15-mer), formed a bimolecular tetraplex in aqueous solution and bound to thrombin with high affinity. In addition, the C15-mer was able to fully displace prebound thrombin from a purified fibrin clot and also inhibited the binding of thrombin to

PARs. Potent in vivo anticoagulant properties of this aptamer have also been demonstrated in animal studies. Nuclear magnetic resonance studies demonstrated that this C15-mer adopted a compact tertiary structure consisting of two tetrads of guanosine base pairs connected by two TT loops and one TGT loop. This chair-like structure is preserved when the C15-mer binds to thrombin. The C15-mer binds to a discrete region within thrombin exosite 1 (fibrinogen recognition exosite), which overlaps the platelet receptor and thrombomodulin binding sites. In our earlier studies, 4-thio-deoxyuridylate-containing oligonucleotides showed high-affinity binding to certain proteins. Therefore, **the other aim of my PhD work was to examine the effects of 4-thio-deoxyuridylate-containing analogs - synthesized in the group of János Aradi - of the thrombin-binding aptamer on different hemostatic/thrombotic activities of thrombin and compared them with the effect of C15-mer.**

Materials and Methods

Preparation of Collagen Matrices and Measuring Binding Efficacy

Human collagen type-III or I was dissolved in 0.05 M acetic acid (pH 2.8) to obtain 2 mg/mL stock solutions. The stock solutions were treated differently: (1) further diluted with 0.05 M acetic acid; (2) diluted with phosphate buffered saline (PBS); (3) diluted with 0.05 M acetic acid and neutralized locally by adding Na_2HPO_4; (4) dialyzed against PBS for 2 days at 4°C. The solutions were added into the wells at serial dilution, and were incubated overnight at 4°C.

To study the effect of pH, collagens were coated from 6 different solutions: sodium acetate 0.02 M, pH 4.0 (1); sodium phosphate 0.02 M, pH 6.4 (2), pH 7.4 (3) and pH 8.0 (4); sodium carbonate 0.02 M, pH 9.0 (5) and acetic acid 0.05 M, pH 2.8 (6); with varying concentration of NaCl. The bound collagen and VWF were detected by specific antibodies.

Atomic Force Microscope (AFM)

Drops of 10 L solutions were put onto glass coverslips and kept in a humid box for 48 h at 4°C. The final concentrations of the collagens were 200 g/mL in acid and 20 g/mL in PBS solutions. Imaging was performed with a custom-made stand-alone-type AFM in tapping mode with samples under water (Zeiss Axiovert microscope).

Blood Collection and Handling

Blood samples were obtained from healthy volunteers who had not taken anti-platelet medication and were anticoagulated with sodium citrate for platelet aggregation studies, clotting time assay, or by low molecular weight heparin for platelet adhesion studies.

Platelet adhesion

Human collagen, diluted in PBS or acetic acid, was layered over thermanox coverslips and incubated in a humid box overnight at 4°C. The coated coverslips were mounted in the wells of an Impact-R, *in vitro* flow chamber (DiaMed) and anticoagulated blood was circulated at a shear rate of 1800 s^{-1} for 2 min. Then the coverslips were analyzed by light microscope.

Scanning Electron Microscope (SEM)

Morphology of the platelets, which had adhered after shear stress onto collagen matrix was studied by SEM. The coverslips were air-dried, gold coated, and examined by a Jeol 840 scanning electron microscope (Jeol USA).

Aptamer Synthesis and Purification

Newly designed aptamers were synthesized in the laboratory of János Aradi, by standard phosphoramidite chemistry, using an automated oligonucleotide synthesizer (Pharmacia Gene-Assembler Plus). Crude products were purified by ion-exchange chromatography and gave a single band by denaturing PAGE.

4-thio-deoxyuridylate (X) was used to replace the T at various positions, see Table 1.

Table 1: The sequences and the names of the consensus aptamer and its 4-thio-deoxyuirdylate modified analogs.

5' → 3' Position of the nucleotide base in the aptamer															
1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**	**14**	**15**	**Short name**
G	G	T	T	G	G	T	G	T	G	G	T	T	G	G	C15
G	G	X	T	G	G	X	G	X	G	G	T	X	G	G	UC15
G	G	X	X	G	G	T	G	T	G	G	X	X	G	G	vUC15
G	G	X	X	G	G	X	G	X	G	G	X	X	G	G	aUC15

The C15-mer was modified via replacing the T at various positions with 4-thio-deoxyuirdylate, represented as X.

Thrombin Clotting Activity Measurement

Fibrinogen solution or pooled normal human plasma was incubated at with Owren's buffer containing various concentrations of aptamers for 1 min prior to the addition of thrombin.. Clotting time was measured by KC-1 coagulometer (Amelung). To test the specificity of the aptamers toward thrombin, in certain experiments thrombin was replaced by reptilase.

Fibrinopeptide A (FpA) Measurement by LC-MS

Thrombin was added to plasma aliquots preincubated without or with increasing concentrations of aptamers as described above. A synthetic internal standard peptide used for quantification was also included in the incubation mixture. All reactions were stopped when plasma sample without aptamer was clotted. FpA was quantified by LC-MS (API 2000).

Amidolytic Assay of Thrombin Activity

The hydrolysis of chromogenic substrate S-2238 by thrombin was measured at 405 nm. Thrombin was added to buffer containing various concentrations of aptamers. The reaction was started by the addition of substrate. In the experiments investigating the effect of UC15-mer on the inhibition of thrombin by hirudin, thrombin activity was measured in the presence of 2.5 nM hirudin and various concentrations of UC15-mer.

Platelet Activation

Platelet activation studies were performed in the presence of GPRP (fibrin polymerization inhibitor peptide, Gly-Pro-Arg-Pro) in a lumiaggregometer (Chrono-log). Thrombin or horm collagen was used as platelet agonist. Aptamers were pre-incubated with PRP (platelet rich plasma) for 1 min prior to the addition of thrombin. The same experiments were also carried out with WPS (washed platelet suspension). The extent of platelet aggregation was quantified by measuring the slopes of aggregation curves. Platelet secretion was monitored simultaneously by measuring the ATP released using luciferin-luciferase reaction. To test the specificity of the aptamers toward thrombin, in certain experiments thrombin was replaced by TRAP-1, thrombin receptor activator peptide that activates the receptor independently of the action of thrombin.

Thrombus Formation at Shear Condition

The effect of aptamers on thrombus formation was studied at a shear rate of 650 s^{-1}, 5 min, using a homemade or Impact-R (DiaMed) cone and plate adhesion device. Glass coverslips were coated with extracellular matrix of HMEC-1(human dermal microvascular endothelial cells) or with “thrombin treated fibrinogen”. Heparinized blood containing various concentrations of C15-mer, UC15-mer or hir54-65 was circulated. Thrombus formation was evaluated by light microscopy.

Results

Coating Conditions

The duration of coating time did not influence the amount of collagen coated as detected by antibody against collagen. On the other hand, the variation of OD-s within each plate was high at 2 and 4 hours coating and low at one or two overnights. As a result of this we used overnight coating for the subsequent experiments.

In order to test how the collagen matrix was affected by the ionic strength of the coating solution, we applied increasing concentrations of NaCl. The OD increased, showing saturation in response to increasing salt concentration, both in the case of the type-I collagen or the type-III. For the subsequent experiments we used PBS containing 0.15 M NaCl unless otherwise stated.

We made serial dilutions of collagen in solutions presenting 6 different pH values in order to see the effect of pH on the matrix preparation. The highest collagen binding was when collagens were diluted in phosphate buffer pH 7.4. The addition of 0.15 M NaCl to the solutions resulted in higher collagen binding to the surface and VWF binding to collagen. Very low binding were for collagen diluted in acid solution.

Binding Characteristics of Collagens from Acid and PBS Solutions

To compare the binding of collagen from acid or from PBS solutions, we designed a series of experiments. Serial dilution of human collagen type-I and type-III with PBS or acetic acid were applied into the wells. Na_2HPO_4 was added to one series of acid diluted collagens to neutralize acid. All the wells were incubated overnight at 4°C. Next day wells were emptied and detection was proceeded with collagen-specific antibody or through VWF binding. Binding of collagen showed a saturation curve from PBS solutions with both detection methods. The binding were low when the surfaces with collagens were coated from acid solution. However, neutralization of the acid with Na_2HPO_4 also resulted in a saturation curve. The same was true of another series of acid-diluted collagens, which after the overnight coating, were transferred into new wells where the acid solution was neutralized. Furthermore, the emptied acid wells were filled with PBS and

left at 4°C for coating as before, but hardly any collagen-specific antibody or VWF binding could be detected.

Correlation of the Collagen Coated and the VWF Captured

When we analyzed the result obtained by measuring the collagen-specific antibody binding and the VWF binding to the coated collagen, we found high correlation with linear regression both for type-I and III collagen.

Morphology of Collagens

AFM was used to test if the quantity different only or the structure also. We found quantitative differences between collagens bound in acid solution or in PBS and collagen fibrils in PBS were more develop than acid. The structure of the collagen type-I was similar to type-III fibrils.

Platelet Adhesion

The plasma protein VWF immediately binds to the fibrillar collagen surface (but not to the acid coated collagen) and platelets adhere to this composed surface. The total amount of platelets adhering to the surface was high for collagen form PBS and low for acid solution. In order to examine the structure of the platelet aggregates on the fibrilar collagen coated surface the sample was examined by SEM. The fibrilar collagen coated surface demonstrated much more "developed" aggregate with fully activated and merging platelets compared to the adhered, spread platelets with filopodia on the plastic surface. The surface coverage of the acidic collagen coated slides was under the limit of the quantitation, and therefore it was not rational to take it for SEM.

Inhibition of Thrombin with Synthetic Oligonucleotides

The Effect of Aptamers on the Clotting and Amidolytic Activity of Thrombin

Exosite 1 is a major fibrinogen recognition site on thrombin. To determine whether the modified aptamers binds to this region and competes with its native substrate, thrombin clotting time assay was performed with purified fibrinogen and human plasma in the presence of the aptamers. C15-mer and UC15-mer inhibited the clotting of purified fibrinogen and plasma by thrombin; aUC15-mer and vUC15-mer were ineffective. In the case of fibrinogen solution the IC50 for UC15-mer was three-fold lower than for C15-mer, in the case of plasma the difference was 2.2-fold. The inhibition of FpA release by thrombin was practically identical with that obtained in clotting time experiments. These assays were repeated with reptilase, none of the aptamers had any effect on this reaction, confirming the thrombin-specific effect of aptamers. The amydolytic effects of thrombin on the chromogenic substrate, S-2238, was not influenced by the aptamers, indicating that the aptamers did not exert their effect on the catalytic center.

Thrombin was also relieved from the inhibitory effect of hirudin by increasing concentration of UC15-mer suggesting that UC15-mer competes with hirudin for thrombin exosite 1.

Effect of Aptamers on Thrombin Induced Platelet Activation

In PRP C15-mer and UC15-mer inhibited aggregation and ATP secretion in dose-dependent manner. In both cases the IC50 values for UC15-mer were only half of the IC50 values for C15-mer. In WPS the IC50 values drastically decreased for both aptamers and UC15-mer became 12-fold more effective than C15-mer. The lack of aptamer effect on TRAP-1 and collagen induced aggregation demonstrates its thrombin specificity.

Effect of Aptamers on Thrombus Formation at Shear Condition

Thrombus formation in whole blood was tested on thrombogenic surfaces in cone and plate chamber. Without aptamers and hir54-65 the HMEC-1 matrix and thrombin treated fibrinogen surfaces were equally covered by large thrombi. When aptamers were present at high inhibitory concentration, both extracellular matrix of HMEC-1 and thrombin treated fibrinogen surfaces

were covered with higher number, but smaller and less thick thrombi and many individual platelets. The effect of aptamers was comparable to the effect of hir54-65. There was no detectable change in thrombus formation on collagen surface in the presence of aptamers.

Discussion

Collagen matrix

Random attachment of different types of collagens leads to a large variation of matrices used by different studies for collagen VWF and platelet interaction. These matrixes mainly depend on the source and preparations of collagens used for coating and have important experimental implications. In the present study, we used human fibrillar collagens type-I and III at varying conditions to prepare collagen matrix for VWF and platelet binding. This kind of systemic study has not been done before.

In the literature, the coating time applied for binding collagen to the wells varies from a few to 72 hours. According to our measurements, the binding of collagens to the surface showed saturating kinetic even at 2 hours coating time. However, with the shortening of the time to less than 12 hours the error of the VWF binding capacity of the matrix increased. Extending the time over this period did not improve its antibody or VWF binding. Moreover, a long coating time may result in a change of the pH of the acid solutions associated with initiation of collagen binding in the wells, resulting in uncontrolled coating condition. Collagen fibril formation is an ongoing process during the standing of solutions, which may influence coating and may explain the result of previous studies.

We found that collagen binding reaches saturation around the physiological salt concentration. It was demonstrated that collagen fibril formation depends on salt concentration. We explain our results, that a higher salt concentration shifts the equilibrium of collagen in different solutions to fibrils, which may bind better to surfaces.

As many studies used collagen dissolved in acetic acid or dialyzed it against PBS, we tested the effect of pH on the coating efficacy of collagen from different solutions. The collagen diluted from acid stock solution to pH 7.4 was superior to those diluted to pH 4.0, 6.4, 8.0, 9.2. Studies on collagen fibril formation showed that neutralize condition is needed to obtain well-ordered fibrils. We can deduct from a series of our experiment that collagen present in acid solution is not in the right binding conformation, because it was coated well after neutralization locally. Adding salt to these solutions to reach the physiological concentration we observed increased collagen coating at all pH-s. It seems that fibril formation and coating efficacy of collagen is a

linked process.

We have found, that incubating the well with PBS after removing the acidic coating solution did not result in normalized VWF binding capacity. In view of these data, we concluded, that the low binding capacity of the acidic coated collagen surface was caused by the low amount of collagen bound to the wells and not because of its inappropriate conformation. Another approach to answer the question was the morphological investigation of the collagen with AFM. This technique revealed fibrillar structures from PBS solutions and from acid too. However, fibrils from acid solution, even under extreme conditions, were considerably fewer, well separated and thinner compared to fibrils from PBS solution.

The extent of platelet adhesion was very low in our study when the collagen was coated from acid solution and it was similar to the results of other studies when the collagen was coated at optimal pH and salt concentration. It is known from real time analysis that fibrillar collagen structure influences the extent and the mechanism of thrombus formation under flow, however, our end point detection technique did not allow the exploration of this kind of difference. As regards the difference in platelet adhesion to collagen coated from acid solution, our explanation is that the collagen binding was partial to the surface, as it was in the 96-well plate. The partial adhesion was feasible due to instant but limited immobilization of both plasma fibrinogen and VWF on the polystyrol surface serving as a thrombogenic substrate but those few adhering platelets were activated but not aggregated.

In summary, our study provides evidences for first time that collagen in acid solution demonstrates limited surface coating resulting in limited and variable VWF binding and platelet adhesion. Neutralizing the acid and adding NaCl in physiological concentration, thereby facilitating formation of collagen fibril molecules in solution, results in efficient coating. In addition, normal VWF binds equally well to human type-I and type III collagens. As collagen-binding activity of VWF is measured routinely when differential diagnosis of VWD is needed, we advise the standardization of the methods regarding to the collagen coating condition.

Thrombin inhibition

The inhibitory effect of UC15-mer was tested on thrombin-catalyzed fibrin clot formation and fibrinopeptide A release from fibrinogen, thrombin-induced platelet aggregation/secretion, and the formation of thrombus on coverslips coated with human collagen type III, thrombin-

treated fibrinogen or subendothelial matrix of human microvascular endothelial cells. The inhibition exerted by the new aptamer was stronger than the inhibition by C15-mer tested by clotting activity. Similar results were obtained when thrombin activity was measured by the release of FpA. The IC50 for both aptamers was higher in plasma than in fibrinogen solution. Specific binding of the aptamers to prothrombin and, perhaps also the less specific matrix effect of other plasma proteins might be responsible for the discrepancy. The effect of plasma proteins on the inhibition by UC15-mer is somewhat more considerable than on the inhibition exerted by C15-mer.

It was shown by chromogenic substrate that UC15-mer, similarly to C15-mer, did not exert its effect through the catalytic site of thrombin. To prove that thrombin exosite 1 was involved in the inhibition of thrombin clotting activity by UC15-mer, we tested the interaction of UC15-mer and hirudin. Hirudin contacts exosite 1 of thrombin through its extended C-terminal tail residues and as a consequence its N-terminal domain occludes the active site blocking both clotting and amydolitic activity. If UC15-mer was bound to exosite 1, it should relieve the inhibition of amidolytic activity. Indeed, as measured by chromogenic substrates, the presence of UC15-mer suspended the inhibitory effect of hirudin.

The 4-thio-deoxyuridylate modification of C15-mer also resulted in a 2-fold more potent inhibition of thrombin induced platelet aggregation and secretion in PRP. TRAP-1-induced platelet aggregation was not influenced even by the highest concentrations of the aptamers, confirming that binding of aptamers to thrombin blocks its ability to cleave PAR-1.

In WPS the aptamers were far more effective than in PRP. The difference between IC50 values measured in PRP and WPS is considerably more than the difference in IC50 values for plasma and fibrinogen clotting. The reason for different effect of plasma proteins on the inhibition of thrombin-induced clotting and platelet aggregation is not clear. It could be due to the 3-fold higher concentration of plasma proteins, including prothrombin, in the experimental set-up for PRP aggregation, than for the plasma clotting. The absence of fibrinogen in WPS could also be a factor. Finally, the concentration of free Ca^{2+} in WPS is about 20-fold higher than in citrated plasma and the effect of citrate on platelet aggregation and on its inhibition by certain platelet antagonists has been demonstrated. The difference between the inhibitory effect of C15-mer and UC15-mer is also much higher in WPS than in PRP; in WPS the IC50 for UC15-mer is one magnitude lower than that for C15-mer.

We also tested the effect of aptamers on thrombus formation. We used HMEC-1 matrix, thrombin-treated fibrinogen and collagen in a flow chamber. In our experiments the large thrombi formed on the matrix of HMEC-1 and thrombin treated fibrinogen was inhibited by both aptamers, but platelet adhesion did not seem to be affected. Like other thrombin specific inhibitors, aptamers did not influence platelet adhesion to collagen. The results suggest that thrombin exosite 1 is involved in the accumulation of platelets into large thrombi on HMEC-1 and thrombin-treated fibrinogen, but not on collagen surfaces.

In summary, the replacement of thymidylate at positions 3, 7, 9, and 13 of the consensus aptamer by 4-thio-deoxyuridylate resulted in more potent inhibition of thrombin-induced fibrin clotting and platelet activation, although the difference in the effectiveness of the two aptamer varied between 2 to 12-fold in different experimental systems. The new aptamer was also more effective in decreasing thrombus deposition on thrombin-treated fibrinogen surface. The replacements introduced two major changes into the physico-chemical characteristics of the molecule. 1/ Due to the tautomer conversion of the modified nucleotide, it may carry (in enol form) reactive –SH group or groups, and this group positioned properly, may interact with –SHs of proteins. Since there is no free –SH on thrombin, this change does not explain the increased inhibitory capacity of UC15-mer. 2/ The thiono group at position 4 renders the base more hydrophobic, which may result in stronger hydrophobic aptamer-protein interactions. We have shown that the homo-oligonucleotide composed of 4-thio-deoxyuridylates is highly resistant to nucleases. Since the UC15-mer contains more than 26% thiolated nucleotide, it should be more stable in biological environment than its unmodified counterparts. This feature may also be of significant advantage in the case of *in vivo* application. Further optimization of 4-thio-deoxyuridylate containing aptamers presents an opportunity for the generation of more potent thrombin inhibiting aptamers with potential use as antithrombotic agents.

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2. **Mendelboum Raviv, S.**, Horváth, A., Aradi, J., Bagoly, Z., Fazakas, F., Batta, Z., Muszbek, L., Hársfalvi, J.: 4-thio-deoxyuridylate modified thrombin aptamer and its inhibitory effect on fibrin clot formation, platelet aggregation and thrombus growth on subendothelial matrix.
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Presentations

- Research Training Network" meeting, Debrecen, Hungary, April 2005. THE EFFECT OF A NEW THROMBIN APTAMER ON PLATLET ACTIVATION.
- "Research Training Network" meeting, Paris, France, September, 2006. COLLAGEN AND VWF INTRACTION. **S.MendelboumRaviv** and J. Harsfalvi
- 19th International Congress on Thrombosis, Tel Aviv, Israel, May 14-18, 2006. THE EFFECT OF A MODIFIED THROMBIN APTAMER ON THROMBIN ACTIVITY AND PLATELET ACTIVATION**. S. Mendelboum**, A.Horvath, J.Aradi, L .Muszbek, J.Harsfalvi.
- 22th International Society of Thrombosis anfHaemostasis, Boston, USA, July 11-16, 2009. EFFECT OF COATING CONDITION ON COLLAGEN MATRIX FORMATION: VON WILLEBRAND FACTOR AND PLATELET BINDING TO IT. **S.MendelboumRaviv**, K Szekeres-Csiki, A. Jenei, J.Nagy, B. Shenkman, N. Savion, J. Harsfalvi
- 33th Word Congress of International Society of Hematology, Jerusalem, Israel, October 10-13, 2010. EFFECT OF COATING CONDITION PN COLLAGEN MATRIX FORMATION: VON WILLEBRAND FACTOR AND PLATELET BINDING. **S. MendelboumRaviv**, K Szekeres-Csiki, A. Jenei, J.Nagy, B. Shenkman, N. Savion, J. Harsfalvi

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