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NOVEL INHIBITORS OF FACTOR Xa REVEALED FROM VIRTUAL SCREENING STUDIES

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Introduction. Cardiovascular related diseases are the major cause of mortality in the developed countries. Intensive biological studies were devoted to the blood coagulation cascade in order to find the key enzymes responsible for the formation of the blood clots. Currently, treatment of strokes or infarctions caused by thromboembolism of vessels is carried out using thrombolytic therapy or by anticoagulant drugs.

Factor X is the calcium-binding, gamma carboxyglutamyl(Gla)-containing, vitamin K dependent glycoprotein [1, p. 241-280]. The activity of Factor Xa is dependent on its inclusion in the prothrombinase complex. The prothrombinase complex converts the prothrombin into the active procoagulant thrombin. It is therefore clear that Factor Xa catalyzes the pre-final step in the blood coagulation cascade, namely the formation of the thrombin. In turn, thrombin cleaves-off fibrinogen fibrinopeptides provoking fibrin formation and self-assembling to fibrin clot. It has been suggested that compounds which selectively inhibit Factor Xa may be useful as *in vitro* diagnostic agents, or can be used as therapeutic agent being administrated in certain thrombotic disorders.

Selective Factor Xa inhibitors decrease risk of bleedings and improved safety/efficiency ratio at the preclinical stages of drug development, in comparison to thrombin inhibitors. The Factor Xa inhibitors are classified as direct and indirect inhibitors depending on the mechanism of action. Direct inhibitors interact directly with the Factor Xa active site and block it. These inhibitors bind efficiently to clot-bound and prothrombinase-associated forms of Factor Xa, unlike the indirect inhibitors. The action mode of indirect inhibitors is based on Factor Xa inactivation by antithrombin [2, P. 671-698].

Methods. The amidolytic activity of Factor Xa was determined by measuring the amount of p-NA released upon hydrolysis of chromogenic substrate. The hydrolysis rate of chromogenic substrate was evaluated by measuring the absorbance at 405 nm at 37 °C at 5 min incubation in Tissue CulturePlate 96-Well Flat Bottom (Sterile) with a microplate reader (Multiscan EX). The reaction mixture in 0.05 M Tris-HCl buffer (pH 7.45) with 0.13 M NaCl and 10^{-3} M $CaCl_2$ with total volume 0.25 ml contained: *for blood plasma*: referent blood plasma – 0.04 ml/ml; Factor Xa activator – 0.02 ml of 1 mg/ml solution – snake venom *Vipera russelli* (RVV) [3, p. 211-217]; inhibitor – $5 \cdot 10^{-6}$ M; S2765 (Z-D-Arg-Gly-Arg-pNA 2HCl) – 0.16 mM; *for purified Factor Xa*: purified bovine Factor Xa (Hemolab Heparichrom) – 0.27 nkat/ml; inhibitor $5 \cdot 10^{-6}$ M, S2765 (Z-D-Arg-Gly-Arg-pNA 2HCl) – 0.16 mM.

The rate of the reaction was measured as the change of absorbance per minute.

For K_i calculation the equation of competitive type inhibition was used:

$K_i = [I]/(K_p/K_m - 1)$, where K_p and K_m – the effective Michaelis constants calculated from Lineweaver-Burk plot at 4 presence and absence of the inhibitors respectively.

Results. All the selected 1400 compounds have been subjected to test with use of RVV-test modified assay (RVVT). We have monitored inhibition activity of the compounds at 5 minutes. We observed the exponential-like regression of the compounds number of along the potency increase (Fig. 1). To assess activity of the most active compounds we selected 59 compounds that have exhibited activity more than 70 % and subjected them to further biological screening on purified Factor Xa. The screening was performed with a 5 μM inhibitor concentration. Most of the selected compounds possess activity ranging from 40 % to 60 %. We selected only 17 compounds that inhibited the activity of Factor Xa on 60-100 % (Fig.).

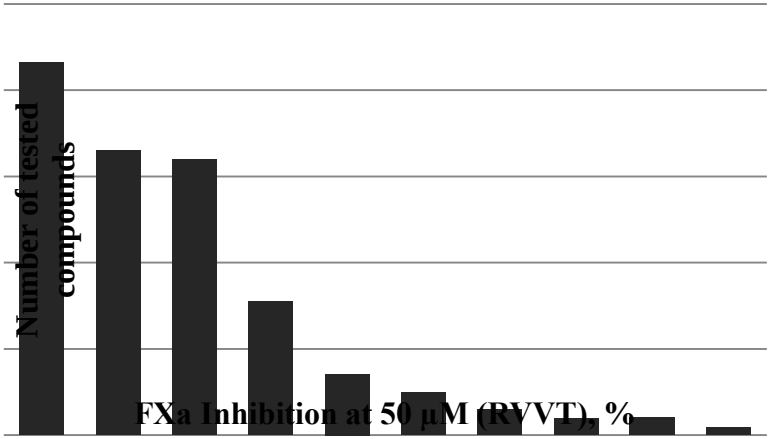

Fig. – Activity distribution of the factor Xa targeted library. Activity distribution of the 1400 compounds subjected to modified RVVT assay at 50 µM inhibitor concentration. Exponential regression is observed.

All samples were tested in 2 model systems as it is described in Materials and methods. The data analysis indicated highest inhibitory activity of two components. The Lineweaver-Burk plot was built according to experimental data to confirm inhibition type and calculate the inhibition constant for the most active inhibitor. The intersection of the curves on the Y-axis suggested the competitive character of inhibition (K_i=13,9·10^{-6} M). This value of the inhibition constant demonstrated the high affinity of the inhibitor to the enzyme.

Conclusions. We used virtual screening to select from the stock virtually generated library those compounds which effectively inhibited Factor Xa activity. The composed set consisted of 1400 compounds that all were subjected to RVVT *in vitro* screening. Two compounds with prominent inhibitory activity towards Factor Xa were selected. Their scaffold easily allows us to synthesize water soluble derivatives without any lose in other properties.

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