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Design, synthesis and anticoagulant studies of new antistasin isoform 2 and 3 amide analogues

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One of the most important enzymes in blood coagulation cascade is Factor Xa. That is why its inhibitors are promising alternative against thrombotic disorders. In our previous work, we reported the synthesis of hybrid structure between isoform 2 and 3 of antistasin and the active sequences D-Phe-Pro-Arg; D-Arg-Gly-Arg; Phe-Ile-Arg and Tyr-Ile-Arg. Besides the analogues with C-terminal COOH group, the peptide D-Phe-Pro-Arg-Pro-Lys-Arg-NH_2 was synthesized. The biological activity of the last one was 60 times bigger than that of the natural isoform 3 of ATS and some times more active than that of the all other synthesized analogues. In the current work, we described synthesis and biological activity of C-terminal amide analogues of all early synthesized peptides in order to deduce the structure-activity relationship. The anticoagulant activity according to the APTT and IC_{50} was determined.

Key words*:* anticoagulant activity, Factor Xa, thrombin, antistasin, peptide mimetics.

INTRODUCTION

During the last years the number of deaths due to hemostatic impairments such as coronary angioplasts, coronary thromboembolisms, myocard heart attack, pulmonary embolism, etc. has become equal to those caused by cancer formations. Haemostasis is a key process whose correct functioning is an important defence mechanism of the human organism. It is a blood coagulation process activated in case of injury of the blood system. If it is functioning correctly, vascular-motor and cell reactions are triggered and the blood coagulation cascade is activated. Coagulation is a defence function of the organism that has to be strictly regulated. After the bleeding is stopped, a number of limiting self-regulatory mechanisms are initiated. They act competitively and their role is to:

- stop further coagulation;
- prevent the thrombus formation in the organism;
- restrict the coagulation to the injured are.

Besides the mechanisms limiting the formation of thrombocytes, the availability of plasma proteins is also important in this respect as they deactivate the serine proteinase of the coagulation, i.e. they act as inhibitors. The main function of blood coagulation is the conversion of the soluble fibrinogen into insoluble fibrin clot. This process is accompanied by a series of enzyme reactions described in 1964 as an enzyme cascade (Fig. 1) [1]

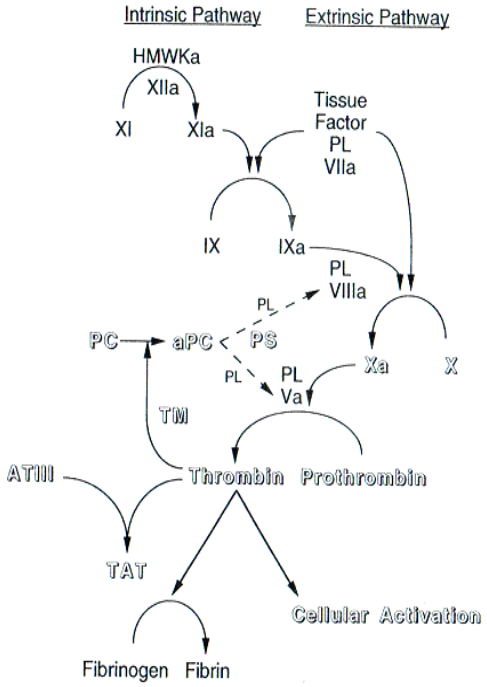

Fig. 1. Scheme of the blood coagulation cascade.

A major role in them plays a number of serine proteinases, which are known as:

- Blood clotting factors
  - extrinsic system: Factor III, Factor VII;
  - intrinsic system: Factor XII, high molecule kininogen, prekalikrein, Factor XI, Factor IX, Factor VIII;
  - common pathway: Factor XI, Factor V, Phospholipids, Factor II, Factor I, Factor XIII
- Factors of the fibrinolysis;
- Factors of the control mechanisms (inhibitors).

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According to the avalanche theory, coagulation can be considered as an auto-bioenhancing process. As a result, the inhibition of proteinases located in the centre of the process would prevent the avalanche formation of strong enzyme substrates down the chain. This thesis is proved by the fact that the inhibition of Factor Xa prevents the activation of 138 thrombin molecules [2]. That is why the main target in the creation of anticoagulants is the inactivation of Factor Xa.

In the last 20 years, a number of proteins and peptides with different molecule mass and well-established anticoagulation activity have been isolated from the salivary glands of several bloodsucking animals. Many of the strongest anticoagulants isolated from bloodsucking animals are found in the saliva of different types of leeches (Table 1).

In 1987 Tuszynski *et al*. reported for 119 amino acids protein isolated form the salivery glands of Mexican leech *Haementeria officinalis* with strong anticoagulant properties, which they named antistasin [64] (Fig. 2).

One year later kinetic investigations of Nutt *et al*. revealed that antistasin is a potent, slow, tight-binding Factor Xa inhibitor [45, 65]. A large part of the natural anticoagulant peptides and proteins isolated later show partial or complete similarity between their active centres and other parts of their molecules and those of antistasin. Thus, it becomes the founder of the largest group of natural anticoagulants – antistasin type inhibitors.

Two years later Condra *et al*. reported that they have isolated three isoforms of antistasin corresponding to its C-terminus which saved good anticoagulant activity [66]:

- isoform 1 /Arg-Pro-Lys-Arg-Lys-Leu-Ile-Pro-Arg/IC_{50} = 5 nM;
- isoform 2 /Arg-Pro-Lys-Arg-Lys/IC_{50} = 500 nM;
- isoform 3 /Arg-Pro-Lys-Arg/IC_{50} = 740 nM.

Recently, the data in literature for short peptides with strong anticoagulant activity increased. A lot of authors publish data for tripeptide sequences as D-Phe-Arg-Pro, D-Arg-Gly-Arg, Tyr-Ile-Arg, Phe-Ile-Arg, which show anticoagulant activity in nanomolar range [51, 57, 58].

EXPERIMENTAL

All compounds were synthesized by standard SPPS by means of Rink amide resin/Fmoc-strategy. The structures were proved by ES/MS.

Table 1. Sources, molecular mass and attacked enzymes of natural inhibitors of the series proteinases published in literature [3–63].

Name of inhibitor	Inhibition activity against different series proteases								Molecular weight, Da	Source
	thrombin	plasmin	trypsin	Chimo-trypsin	Plasma calicrein	Tissuae calicrein	elastase	Factor Xa		
AT III	+	ND	ND	ND	ND	ND	ND	+	65000	Blood plasma
haemadin	+	-	-	-	-	-	-	-	5000	*Haemadipsa sylvestris*
draculliND	-	-	-	-	-	-	-	+	83000	*Desmodus rotundus*
TAP	-	-	-	-	-	-	-	+	6977	*Ornithodorus moubata*
savignin	+	-	-	-	-	-	-	-	12430	*Ornithodorus savignyi*
AcAP	ND	ND	ND	ND	ND	ND	ND	+	8697	*Ancylostoma caninum*
anophelin	+	ND	ND	ND	ND	ND	ND	ND	6500	*Anopheles abimanus*
Hirudin	+	ND	ND	ND	ND	ND	ND	ND	ND	*Hirudo medicinalis*
bdellastasin	-	+	+	-	-	-	-	-	6333	*Hirudo medicinalis*
hirostasin	-	-	-	-	-	+	-	-	ND	*Hirudo medicinalis*
madanins	+	ND	ND	ND	ND	ND	ND	ND	7000	*Haemaphysalis longicornis*
therostasin	-	-	+	-	-	-	-	+	8990	*Theromyzon tessulatum*
theromin	+	-	-	-	-	-	-	-	7215	*Theromyzon tessulatum*
therin	-	-	+	-	-	-	-	-	5376	*Theromyzon tessulatum*
rhodniin	+	ND	ND	ND	ND	ND	ND	ND	ND	*Rhodnius prolixus*
lefaxin	ND	ND	ND	ND	ND	ND	ND	+	30000	*Haementeria depressa*
ixolaris	ND	-	-	-	ND	ND	ND	-	ND	*Ixodes scapularis*
guamerin	ND	ND	ND	ND	ND	ND	+	-	6110	*Hirudo nipponia*
pyguamerin	-	+	+	+	+	+	-	-	5090	*Hirudo nipponia*
ekotin	-	-	+	+	-	-	+	+	36000	*Escherichia coli*
ATS	-	-	+	-	-	-	-	+	15000	*Haementeria officinalis*
ghilantens	-	-	+	-	-	-	-	+	15000	*Haementeria ghilianii*

* 2.6 μM plasma concentration is enough to NDutralized reserve of proteins of factor Xa and thrombin.
ND – not determined.

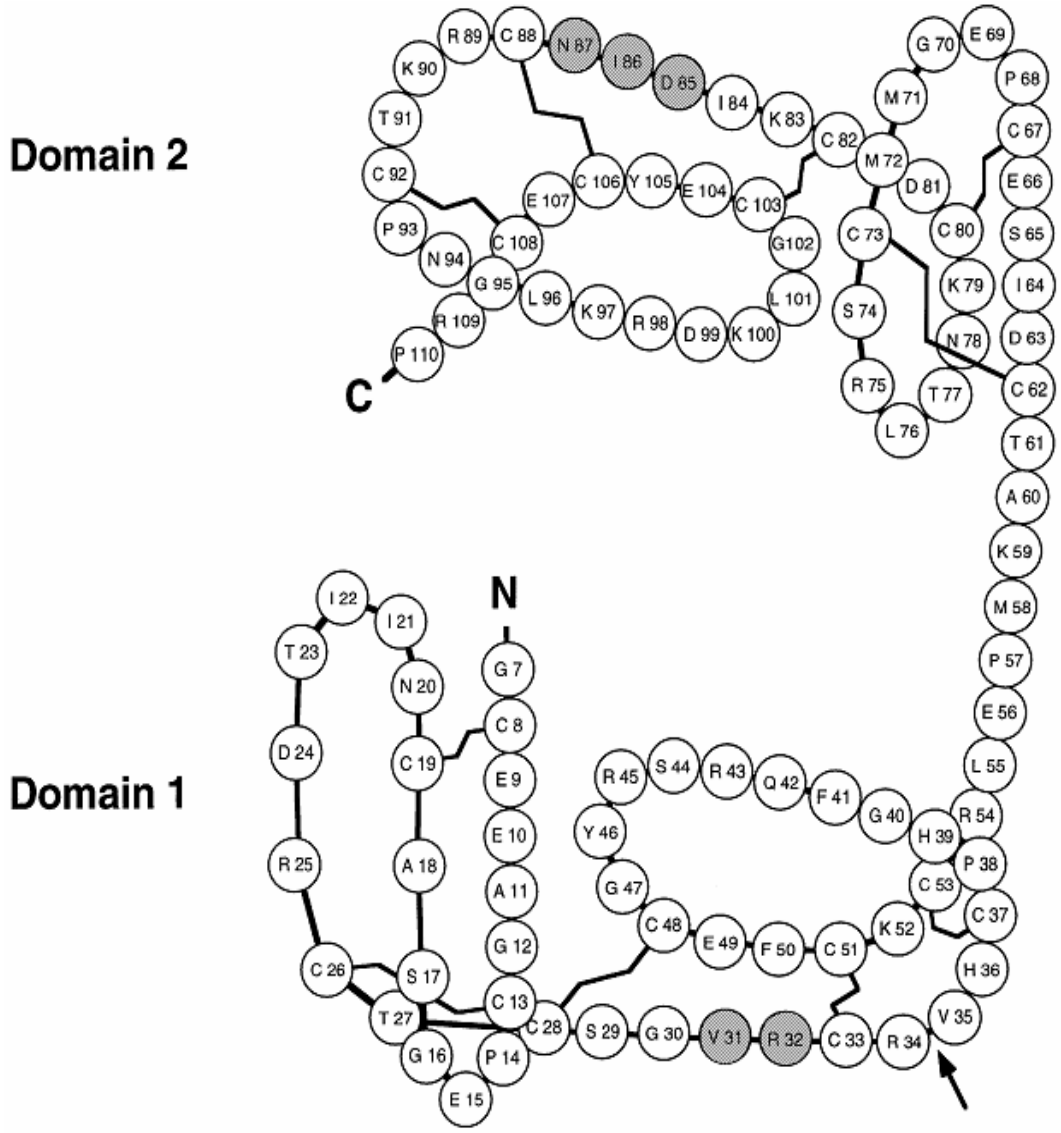

Fig. 2. Primary structure of antistasin.

RESULTS AND DISCUSSION

In 2004 we synthesized and characterized a series of hybrid structures between isoform 2 and 3 of antistasin and the above mentioned tripeptides [59]. The aim of these compounds creation was to obtain new structures with better anticoagulant activity and good selectivity towards different enzymes included in the blood coagulation cascade as well as to reveal the role of some amino acids in the different positions of the molecule. We have established interesting structure-activity relationships. In the same work, we reported for the synthesis of one hexapeptide replacing its C-terminal COOH function with $CONH_2$: D-Phe-Pro-Arg-Pro-Lys-Arg-NH_2. The latter showed 12 times better activity than its analogues with C-terminal COOH group and 40 times higher activity than the natural isoform 3 of antistasin (IC_{50} = 60.6 nmoles).

Based on the fact mentioned above, herein we reported the synthesis of all previously obtained hybride structures but replacing their C-terminal COOH function with amide:

Tyr-Ile-Arg-Pro-Lys-Arg-NH_2
Tyr-Ile-Arg-Pro-Lys-Arg-Lys-NH_2
Phe-Ile-Arg-Pro-Lys-Arg-NH_2
Phe-Ile-Arg-Pro-Lys-Arg-Lys-NH_2
D-Arg-Gly-Arg-Pro-Lys-Arg-NH_2
D-Arg-Gly-Arg-Pro-Lys-Arg-Lys-NH_2.

The anticoagulant activity according to APTT and IC_{50} values were determined. All newly synthesized peptides have manyfold higher activity than the natural isoforms of antistasin. Their IC_{50} are in nanomolar range. The kinetic investigations on the enzymes from blood coagulation cascades are in progress in order to determine the specificity of new hybride structures. The toxicity studies are in progress, too.

CONCLUSIONS

1) Lys^{113} is very important for the substrate-enzyme interaction.

2) The replacement of C-terminal COOH with $CONH_2$ results in manifold increasing of anti-coagulant activity.

3) The replacement of L- with D-amino acid in P_3 position is not a key factor for increasing the anticoagulant activity.

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ДИЗАЙН, СИНТЕЗ И АНТИКОАГУЛАНТНИ ИЗСЛЕДВАНИЯ НА НОВИ АМИДНИ АНАЛОЗИ НА ИЗОФОРМИ 2 И 3 НА АНТИСТАЗИН

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(Резюме)

Един от най-важните ензими в каскадата на кръвната коагулация е Фактор Ха. Ето защо неговите инхибитори са една обещаваща алтернатива срещу тромботични заболявания. В предишна наша работа ние докладвахме синезата на хибридни структури между изоформи 2 и 3 на антистазина и активните последователности D-Phe-Pro-Arg; D-Arg-Gly-Arg; Phe-Ile-Arg и Tyr-Ile-Arg. Освен аналозите с С-крайна COOH група, беше синтезиран и хексапептид амида D-Phe-Pro-Arg-Pro-Lys-Arg-NH_2. Той показа 60 пъти по-висока биологична активност от природната изоформа 3 на антистазина и няколко пъти по-висока активност от всички останали новосинтезирани аналози. В настоящата работа ние описваме синтеза и биологичната активност на С-крайните амидни аналози на всички по-рано синтезирани пептиди с цел да изведем зависимости структура-биологична активност. Антикоагулантната активност беше измерена по отношение на APTT и бяха определени IC_{50} стойностите за всички новосинтезирани съединения.