# Inhibitors of Serine Proteinases from Blood Coagulation Cascade – View on Current Developments

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**Abstract:** The importance of Factor Xa and thrombin in thrombosis and haemostasis is widely appreciated in the last years. This understanding logically leads to the concept of Factor Xa and thrombin inhibition as viable and attractive target for the antithrombotic therapy. The following review collects and summarizes information on numerous anticoagulant peptides, proteins, chemical compounds and low molecular weight fragment analogues of natural peptides isolated from hematophagous. Unfortunately, not much data from clinical trials of direct Factor Xa and thrombin inhibitors is available in the present moment. The current information reveals that Factor Xa and thrombin inhibition is a promising approach for prevention and treatment of hemostatic impairments. The synthesis of a low molecular weight synthetic peptide mimetics could be a key point in the fight against many diseases related to haemostatic disorders.

**Keywords:** Anticoagulant peptides, haemostasis, Factor Xa, thrombin, peptide inhibitors of serine proteinases, blood coagulation cascade.

## INTRODUCTION

The rate of death related to haemostatic impairments such as arterial and venous thrombosis, heart attacks, strokes, and peripheral vascular diseases has reached the rates of death caused by cancer formations in the last decade. As a basic part of haemostasis, the blood coagulation process is responsible for protecting the body from uncontrolled blood loss after injury on the body surface. Its normal function has a very important defence mechanism. This process of blood stopping is started in case of injuring the blood vessel and if the blood coagulation cascade is activated. The blood coagulation is a defence mechanism for the living organism, but only when it is strictly controlled. After ensuring that the bleeding stops, a number of restrictive action mechanisms of self-regulation start as well as activation of the secretion of plasma proteins that inactivate serine proteinases responsible for the clotting process. They act competitively and their role is:

- to stop further spread of coagulation process in blood circulation
- to avoid thrombus formation in the body
- to limit the bleeding in the injury.

A long with the mechanisms limiting the formation of a platelet thrombus, the presence of plasma proteins that inactivate serine proteinases of coagulation (their inhibitors) is also important. The lack of coordination between the process of coagulation and its inactivation, thrombolytic process and other physiological disorders can lead to thrombosis and coagulation problems. The main function of the blood coagulation process is the conversion of soluble fibrinogen to insoluble fibrin network. This process is attends by a series of enzymatic reactions described in 1964 as an enzymatic cascade (Fig. **1**) [1].

In the blood coagulation cascade the central role is played by many serine proteinases known as:

- Coagulation factors
  - external system: factor III, factor VII
  - internal system: Factor XII, high molecular weight kininogen, prekalikrein, Factor XI, Factor IX, Factor VIII
  - common path: Factor X, Factor V, phospholipids, Factor II, Factor I, Factor XIII
- Factors of fibrinolysis
- Control factors (including inhibitors of coagulation process)

Natural mechanisms exist in the living organisms that defeat human body from the clotting and thrombolytic processes, and other physiological disorders. These are implemented through various compounds with a protective function named antithrombotics and include inhibitors of thrombin, factor Xa and other factors involved in the external and the internal pathways of blood coagulation cascade presented above [2].

The main enzymes attracting the most attention in scientific literature and research are the factor X and thrombin because of their key role in the blood coagulation cascade. Therefore, factor Xa and thrombin are representing an excellent target for new antithrombotic therapies.

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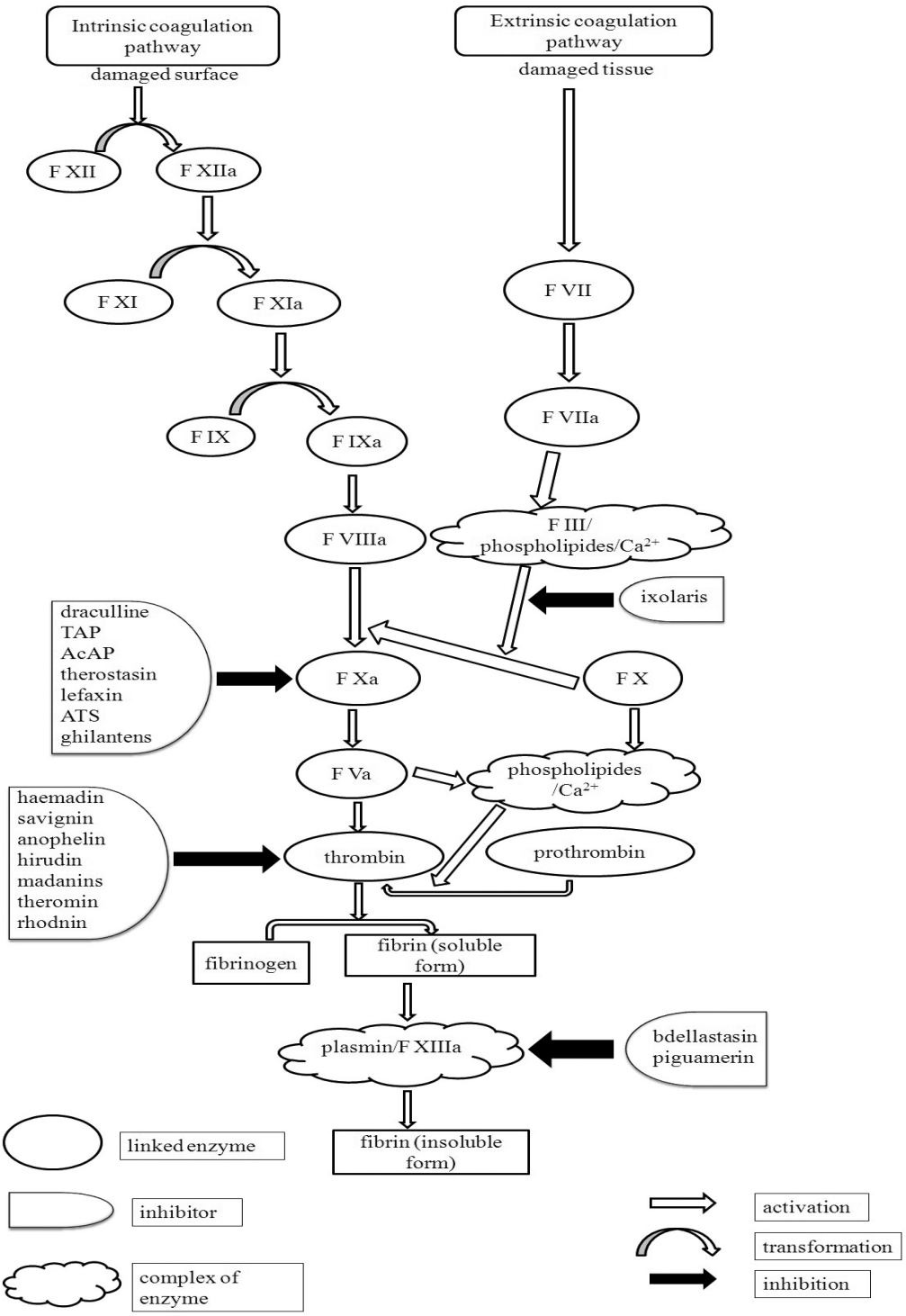


**Fig. (1).** Blood coagulation cascade and places of inhibition of some proteins isolated from bloodsucking animals.

Factor X (Stuart-Prower factor) or autothrombin III is vitamin K dependent serine proeteinase involved in blood coagulation cascade by activating the conversion of prothrombin to thrombin.

It is a glycoprotein dimer (the only one in a prothrombin group with dimeric structure) with a molecular weight of 59 000 Da. The active part is in the heavy chain, which acquires the function of factor Xa after cleavage of low molecular weight fragment. An important feature of factor Xa is that it occupies the central position in the relationship between external and internal coagulation pathways.

Thrombin is a "trypsin-like" serine proteinase protein encoded by the F2 gene in human beings [3,4]. Thrombin in turn acts as a serine proteinase that converts soluble

fibrinogen into insoluble strands of fibrin, as well as catalyzing many other coagulation-related reactions. The functions of thrombin include three levels of vascular control, namely the stationary cell walls, circulating blood cells and liquid phase plasma components. Although the enzymatic production of thrombin from prothrombinase complex, which is indispensable for coagulation, is a collective process, not all coagulation proteins play a key role in the process. In fact, factor X has a major role as its presence increases by approximately 300 000 times the production of thrombin when added to a plasma system containing the other necessary ingredients for prothrombinase complex.

Prothrombin (coagulation factor II) is proteolytically cleaved to form thrombin in the first step of the coagulation cascade, which ultimately results in the stemming of blood loss. As most plasma proteins the prothrombin is synthesized in liver parenchyma and like factor X, it is also a vitamin K dependent protein. Kisiel *et al*. demonstrate a high degree of homology among the vitamin K dependent proteins in the human body. These all have similar structure in their N-and C-terminal fragments [5].

Several reviews have covered different aspects of haemostasis during the years, as well as various inhibitors of factor X and thrombin [6-13]. Only this one published by Clyne *et al*. is summarizing briefly the literature data described to 1998 on natural and synthetic inhibitors of blood coagulation. The other reviews only reveal some aspects of the coagulation process and possible strategies for overcoming some problems in that field. In his review,

**Table 1. Sources, Molecular Weight and Attacked Enzymes of the Natural Inhibitors of the Serine Proteinases Published in the Literature**

| Inhibitor | Inhibition activity against different serine proteases | | | | | | | | Molecular weight (Da) | Source | Reference |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | thrombin | plasmin | trypsin | chimotrypsin | Plasma calicrein | Tissuae calicrein | elastase | Factor Xa | | | |
| haemadin | + | - | - | - | - | - | - | - | 5 000 | *Haemadipsa sylvestris* | [21] |
| draculline | - | - | - | - | - | - | - | + | 83 000 | *Desmodus rotundus* | [22,23] |
| TAP | - | - | - | - | - | - | - | + | 6 977 | *Ornithodorus moubata* | [24,25] |
| savignin | + | - | - | - | - | - | - | - | 12 430 | *Ornithodorus savignyi* | [26] |
| AcAP | ND | ND | ND | ND | ND | ND | ND | + | 8 697 | *Ancylostoma caninum* | [27,28] |
| anophelin | + | ND | ND | ND | ND | ND | ND | ND | 6 500 | *Anopheles abimanus* | [29] |
| Hirudin | + | ND | ND | ND | ND | ND | ND | ND | ND | *Hirudo medicinalis* | [30,31] |
| bdellastasin | - | + | + | - | - | - | - | - | 6 333 | *Hirudo medicinalis* | [26] |
| hirostasin | - | - | - | - | - | + | - | - | ND | *Hirudo medicinalis* | [26,32,33] |
| madanins | + | ND | ND | ND | ND | ND | ND | ND | 7 000 | *Haemaphysalis longicornis* | [34] |
| therostasin | - | - | + | - | - | - | - | + | 8 990 | *Theromyzon tessulatum* | [35] |
| theromin | + | - | - | - | - | - | - | - | 7 215 | *Theromyzon tessulatum* | [36] |
| therin | - | - | + | - | - | - | - | - | 5 376 | *Theromyzon tessulatum* | [37] |
| rhodnin | + | ND | ND | ND | ND | ND | ND | ND | ND | *Rhodnius prolixus* | [38-42] |
| lefaxin | ND | ND | ND | ND | ND | ND | ND | + | 30 000 | *Haementeria depressa* | [14] |
| ixolaris | ND | - | - | - | ND | ND | ND | IAFX** | ND | *Ixodes scapularis* | [43] |
| guamerin | ND | ND | ND | ND | ND | ND | + | ND | 6 110 | *Hirudo nipponia* | [44,45] |
| pyguamerin | - | + | + | + | + | + | - | - | 5 090 | *Hirudo nipponia* | [46] |
| Antistasin (ATS) | - | - | + | - | - | - | - | + | 15 000 | *Haementeria officinalis* | [47] |
| Ghilantens | - | - | + | - | - | - | - | + | 15 000 | *Haementeria ghilianii* | [48] |

*2.6 μM plasma concentration is enough to neutralized reserve of proteins of factor Xa and thrombin; ** IAFX – inhibit activation of factor X; ND – not determined.

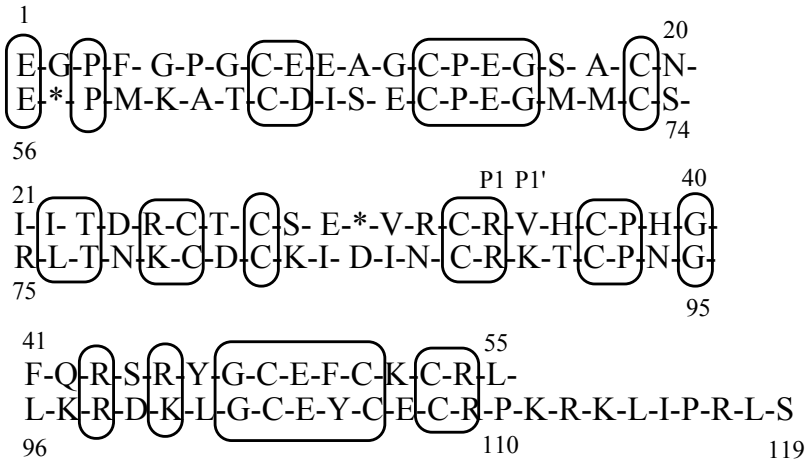


**Fig. (2).** Amino acid sequence of antistasin (ATS).

Alban described the main phases of blood coagulation process and the role of some enzyme in this process [10]. Although, the key role of factor X and thrombin in a blood coagulation process is well proven and they are the main targets for design of new anticoagulants, some inhibitors and other factors of blood coagulation cascade is also described [14-18]. Therefore, the main objective of the present review is to collect and summarize the data in the scientific literature related to inhibitors of coagulation process and especially those with protein (peptide) nature, isolated from bloodsucking animals.

## INHIBITORS OF SERINE PROTEINASES FROM BLOOD COAGULATION CASCADE ISOLATED FROM BLOODSUCKING ANIMALS

The natural defense from hyper coagulation is carried out by antithrombin III (AT III), protein C/S system, heparin factor II and tissue factor pathway inhibitor (TFPI). The first two proteins act as serine proteinases inactivator of coagulation after linking in equimolar ratio. The most important of these hemostatic regulators are AT III and TFPI. Serine proteinases inhibitors represent a large class of proteins, which in structure and mechanism of action are divided into several families. Most inhibitors of coagulation factors of the internal pathway are members of the *serpin* family. In the same time extrinsic pathway inhibitors such as hirudin and antistasin are classified as part of the family of Kunitz type inhibitors [19].

A lot of proteins and peptides with different molecular weight and well established anticoagulant activity are isolated from salivary glands of several bloodsucking animals (teaks, leaches and vampire bat) in the last 30 years.

The initial systematic studies date from the 50's of last century. Markwardt described the activity of a protein in 1955, which he called hirudin [20]. Table **1** summarizes a large number of inhibitors of serine proteinases of blood coagulation cascade, isolated from bloodsucking animals that have been published in scientific literature.

A special place in the literature is given to a protein known as antistasin (ATS) (Fig. **2**).

In 1988 Nutt *et al*. reported isolation from the salivary glands of the Mexican leech, *Haementeria officinalis* and has characterized a protein with high anticoagulant properties which was named ATS [49-51]. Lappato *et al*. published the X-ray structure of antistasin and its modeled complex with factor Xa [52] ten years later in 1998. Three closely related forms of ATS are isolated from the glandular extract (Table **2**) [53]. Recombinant antistasin (rATS) is obtained by recombinant technique using insect, yeast and microbial cells (Table **2**) [45]. These ATSs differ according to natural ATS by C-terminal sequence.

The revealed values of $EC_{50}$ show that C-terminus of ATS has a key role in the inhibition activity.

The kinetic studies reveal that antistasin is a potent, slow, tight-binding Factor Xa inhibitor [54-56]. Many of the later isolated natural anticoagulant proteins and peptides showed partial or complete similarity of the active centers and other parts of the molecules with ATS. Thus it appears to be the

**Table 2. rATS Obtained Using Different Sources**

| Source | C-terminal sequence | $EC_{50}$(nM)* |
|---|---|---|
| insect rATS | Arg-Pro-Lys-Arg-Lys-Leu-Ile-Pro-Arg-Leu-Ser | 3 |
| yeast rATS | Arg-Pro-Lys-Arg-Lys-Leu-Ile-Pro | 20 |
| isoform 1 | Arg-Pro-Lys-Arg-Lys-Leu-Ile-Pro-Arg | 5 |
| isoform 2 | Arg-Pro-Lys-Arg-Lys | 500 |
| isoform 3 | Arg-Pro-Lys-Arg | 740 |

*$EC_{50}$ is a concentration for doubling APTT (activated partial thromboplastin time) in pure platelet plasma.

founder of the largest group of natural anticoagulants - antistasin type inhibitors.

## SYNTHETIC INHIBITOR OF SERINE PROTEINASES FROM BLOOD COAGULATION CASCADE

Contrary to many claims that diseases caused by haemostatic disorders are overtaken by the plagues of the twentieth century(HIV and cancer), the biggest scourge of the new century are obesity and stress. These in turn lead to a large number of deaths caused by the appearance of haemostatic disorders in the body. The need to search for new anticoagulant agents with improved pharmacological properties is a very important solution addressing these issues.

The high molecular weight of the natural peptides is associated with many and different problems in their synthesis for pharmaceutical needs, and a number of difficulties in their isolation, such as purification from natural sources and introducing them in the human body. Therefore, in parallel with the isolation of natural anticoagulants efforts are invested in research for the synthesis of low molecular weight compounds. They must have similar activity combined with shortened sequences of natural peptides or proteins. Moreover, they have to preserve, sometimes even increase anticoagulant activity of natural product which mimic. The literature abounds in evidence of this kind of compounds. Only a few years after 1955 when Markwardt has described the first isolated anticoagulant protein hirudin, the first reports of low molecular weight synthetic inhibitors of serine proteinases on the basis of benzylamine and benzamidine [57] has appeared. Significant data on synthesis and the characterization of many low molecular weight anticoagulants has been published in the last 30 years [58-70]. The most interesting thrombin inhibitors are described in [71]; synthesis and some investigation on potent and selective inhibitors of Factor Xa are published in [72-75].

Over the last decade intensive work was carried out on peptide fragments with anticoagulant activity and shortened analogues of natural proteins with preserved although reduced activity. These are named "peptide mimetics", because they mimic the structure and properties of natural proteins. Peptide mimetics generally have several advantages over synthetic compounds based on non-peptide structures. Basic advantages are the lack of toxicity and accumulation in the body and the easy penetration through cell membranes. The oldest systematic data in the literature about anticoagulant structures based on amino acids and peptides is from 1975, when Bajusz *et al*. reported work on the tripeptide aldehyde D-Phe-Arg-Pro-H [76]. This sequence shows very encouraging results *in vivo*, leading to a number of researches on its modifications [77-79]. Nearly 10 years later, Ostrem *et al*. have described the synthesis and biological activity of new tripeptides Tyr-Ile-Arg and Phe-Ile-Arg, which has shown activities similar to those described earlier for the peptide D-Phe-Arg-Pro-H [80]. At approximately the same time Marlowe *et al*. have described their research on a new peptide D-Arg-Gly-Arg with high anticoagulant activity [81]. During the years many modifications have been made to peptide sequences obtained with high anticoagulant activity mainly related to:

- investigating the role of individual amino acids, including in peptide sequences, in the process of inhibition of enzymes of the coagulation cascade
- achieving selectivity with respect to one or another serine proteinase involved in blood coagulation cascade.

In 1990 Kettner *et al*. and in 1997 Rupin *et al*. have reported a series of boropeptide thrombin inhibitors based upon the D-Phe-Pro-Arg sequence [82,83]. At the same time, Angliker *et al*. have described the synthesis and characterization of thrombin inhibitors based on peptidyl halomethanes [84]. Cheng *et al*. have ascribed a series of phosphonopeptides as a new class of thrombin inhibitors [85].

There is less data on the synthesis of longer structures with similar activity. In 1991, Brancamp *et al*. published a report on their investigations on ghilanten-related inhibitors [86]. In a series of publications our group described the synthesis of fragment analogues of ATS and ghilantens [87-95]. We have investigated the role of basic and D-amino acids in different positions of the molecule on the anticoagulant activity of compounds. We have also synthesized a series of hybrid structures between isoforms of ATS and ghilantens by incorporation of the aforementioned active tripeptides in order to increase the activity of the fragments. We have made important conclusions about structure-activity relationships of these analogues of natural proteins. As a major trend we have observed that the presence of basic and D-amino acids in certain positions of peptide fragments, and the replacement of C-terminal COOH function with the $CONH_2$, increase significantly the anticoagulant activity in some cases.

Some synthetic molecules are described in the literature to be in clinical phase of studies. These react as direct (direct inhibitors are a class of medication that act as anticoagulants (delaying blood clotting) by directly inhibiting some enzyme) or indirect inhibitors (they have the ability to bind to other molecules and cause specific changes to turn them into inhibitors or to enhance their inhibitory potential) of thrombin or factor Xa [96]. According to their chemical nature, these can be subdivided to inhibitors with protein (peptide) origin, with carbohydrate nature and low molecular synthetic inhibitors.

Hirudin and its derivatives (previously known as hirulog) are first discovered inhibitor with peptide nature reached clinical trials [97,98]. Lepirudin (known as Refludan) and Desirudin (Revasc/Iprivask) are recombinant hirudins. They both are almost identical to hirudin extracted from *Hirudo medicinalis,* derived from yeast cells. The first one differs by the substitution of Leu for Ile at the N-terminus of the molecule and the absence of a sulfate group on the Tyr63 [99]. Desirudin is a single-chain polypeptide consisting of 65 amino acids containing 3 disulphide bridges. It differs by the hirudin by lack of sulphate group on amino acid Tyr63. Bivalirudin (Angiomax or Angiox, manufactured by The Medicines Company) is a specific and reversible direct

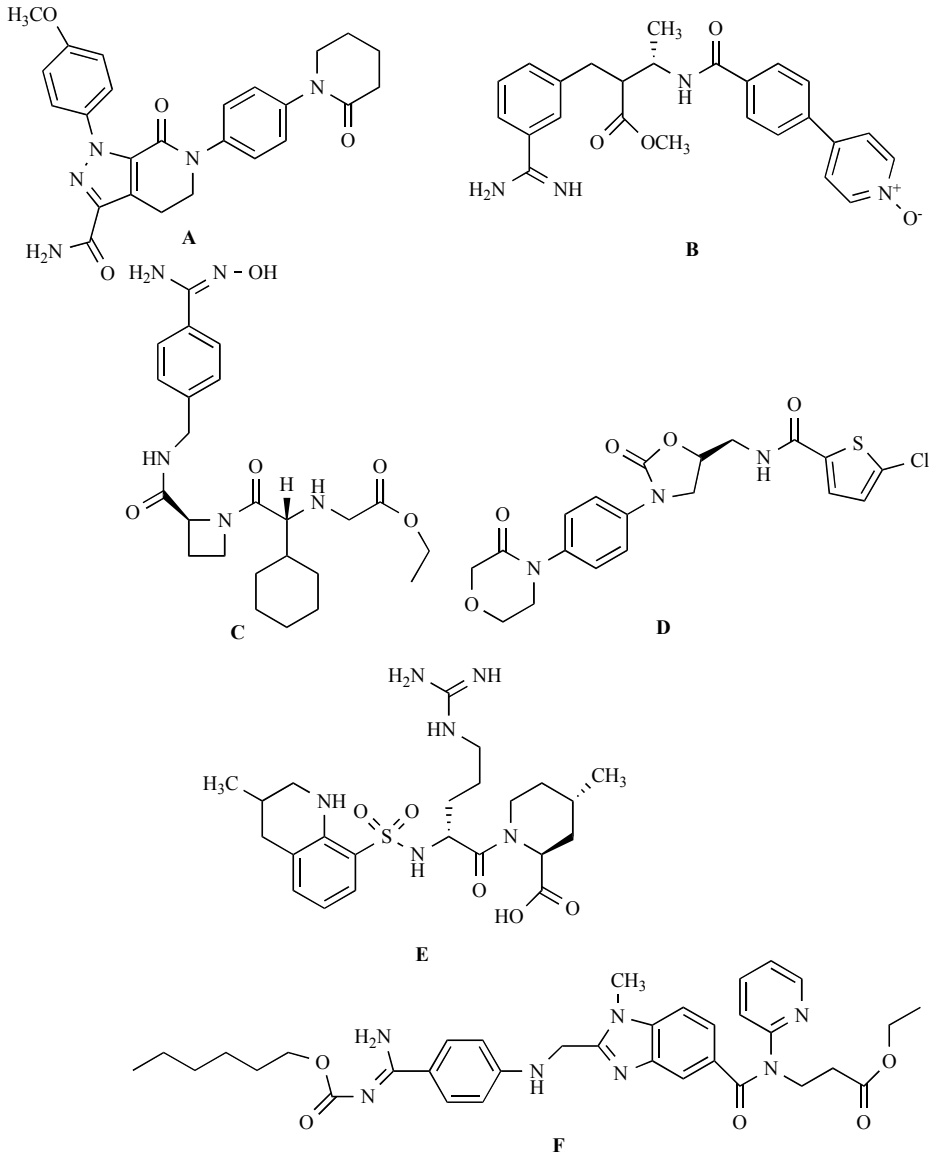


**Fig. (3).** Chemical structure of Apixaban (**A**), Otamixaban (**B**), Ximelagatran (**C**), Rivaroxaban(**D**), Argatroban (**E**) and Dabigatran etexilate (**F**).

thrombin inhibitor [100]. Chemically, it is a synthetic congener of the naturally occurring drug hirudin. Bivalirudin overcomes many limitations seen in indirect thrombin inhibitors, such as heparin and warfarin. Bivalirudin is a short, synthetic peptide that is potent, highly specific, and a reversible inhibitor of thrombin. Bivalirudin has a quick onset of action and a short half-life.

Heparin (both UFH and LMWHs) is the most commonly used in a medical practice representative of the group of anticoagulants with carbohydrate structure. It is isolated by McLean in 1916, and currently it is mainly obtained through extraction and hydrolysis from porcine mucosa. In the present time it is well known that heparin is an indirect inhibitor of serine proteinases thtombin and Factor Xa, It has the ability to bind with antithrombin (AT) making some specific conformational changes in AT molecule which alter the structure of the reactive center of the inhibitor [101]. Unfortunately, heparin and LMWHs have several well-known side effects, e.g. bleeding and heparin-induced thrombocytopenia and the science community is working with a tremendous effort to develop a better anticoagulants. Today some other synthetic polysaccharides are also known and show a very good inhibitory activity. Fondaparinux (trade name Arixtra, marketed by GlaxoSmithKline) is an anticoagulant medication chemically related to LMWHs. It is an indirect selective to Factor Xa inhibitor because he binds to AT. Fondaparinux, like heparins, induces conformational changes in antithrombin molecule that increases the rate of its binding to factor Xa about 300 times [102,103]. Idraparinux is an anticoagulant medication in development by Sanofi-Aventis. It has a similar chemical structure and the same method of action as fondaparinux, but has elimination half-life about five to six times longer (an increase from fondaparinux's 17 hours to approximately 80 hours), which means that the drug should only need to be injected once a

week [104]. Both fondaparinux and idraparinux are selective factor Xa inhibitors.

Some other molecules with non peptide structure are currently in phase III of clinical studies: rivaroxaban (in a number of countries it is marketed as Xarelto, invented and manufactured by Bayer) [105], apixaban (trade name Eliquis, developed in a joint venture by Pfizer and Bristol-Myers Squibb) [106], otamixaban (developed by the French pharmaceutical company Sanofi-Aventis) [107], dabigatran (Pradaxa in Australia, Europe and USA, Pradax in Canada, Prazaxa in Japan) [108], ximelagatran (Exanta or Exarta, manufactured by AstraZeneca) [109] and argatroban [96,110] (Fig. **3**). They are proven direct factor Xa inhibitors. Some of these are derivatives of benzamidin, which is well known competitive inhibitor of many trypsin-like serine proteinases including factor Xa [111].

There are several patents, which prove the practical interest in this topic, too [112-118].

## KINETIC INVESTIGATIONS

According to their mechanism of binding to serine proteinases anticoagulants are direct or indirect (i.e. AT-dependent), and the first group could be subdivided into bivalent or univalent. The thrombin inhibitors can block enzyme action by binding to three different domains:

- thrombin active site
- thrombin catalytic site
- or two exosites

Since bivalent direct thrombin inhibitors bind simultaneously to the active site and exosite 1 of thrombin, univalent direct inhibitors bind only the active site of the thrombin. The phenomenon that heparin can bridge thrombin to fibrin by simultaneously binding both of them is also revealed. Unlike the direct and indirect thrombin inhibitors which differ in the manner of inhibition of serine proteinase, the same two types inhibitors of factor Xa did not differ in their mechanism of binding to the enzyme. The inhibition of factor Xa by both inhibitors is due to their competition with prothrombin for a place in the active site of a serine proteinase. In this case their inhibitory potential depends on the molar concentration of the inhibitor, its affinity to the active site, and the association rate. There are a lot of papers associated with different investigations that determine some important constants for investigation of the type of inhibition of the newly synthesized molecules like Ki, Km, Vmax, $EC_{50}$, etc. [119-128].

## CONCLUSION

In the last couple of years the stressful daily life and the dynamic lifestyle both in the countries from Western Europe and in the developing Eastern Europe have brought to a rapid increase of death caused by haemostatic disorders. This article has summarized all data in the literature related to the anticoagulant agents revealing that the inhibitors of serine proteinases from blood coagulation cascade are a promising alternative for prevention and treatment of hemostatic impairments.

## CONFLICT OF INTEREST

None declared.

## ACKNOWLEDGEMENT

None declared.

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