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Progress of thrombus formation and research on the structure-activity relationship for antithrombotic drugs

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Abstract Many populations suffer from thrombotic disorders such as stroke, myocardial infarction, unstable angina and thromboembolic disease. Thrombus is one of the major threatening factors to human health and the prevalence of cardio-cerebrovascular diseases induced by thrombus is growing worldwide, even some persons got rare and severe blood clots after receiving the AstraZeneca COVID vaccine unexpectedly. In terms of mechanism of thrombosis, antithrombotic drugs have been divided into three categories including anticoagulants, platelet inhibitors and fibrinolytics. Nowadays, a large number of new compounds possessing antithrombotic activities are emerging in an effort to remove the inevitable drawbacks of previously approved drugs such as the high risk of bleeding, a slow onset of action and a narrow therapeutic window. In this review, we describe the causes and mechanisms of thrombus formation firstly, and then summarize these reported active compounds as potential antithrombotic candidates based on their respective mechanism, hoping to promote the development of more effective bioactive molecules for treating thrombotic disorders.

Keywords: Thrombus; Cardio-cerebrovascular diseases; Antithrombotic drugs

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Abbreviations

AA: arachidonic acid	LMWHs: low molecular weight heparins
A3P5P: adenosine 3',5'-diphosphate	MD: molecular dynamics
APC: activate protein C	NO: nitric oxide
AIR: aggregation inhibition rate	NSCS: N-succinyl-chitosan
APTT: activated partial thromboplastin time	NOSCS: N, O-succinyl-chitosan
ASA: acetylsalicylic acid	PA: platelet aggregation
AV-SHUNT: arteriovenous shunt	PAF: platelet activating factor
Ca ²⁺ : calcium ion	PAI-1: plasminogen activator inhibitor -1
COX: cyclooxygenase	PAR: protease activated receptor
CS: Chondroitin sulfate	PDE3: phosphodiesterases 3
sCS: sulfated chondroitin sulfate	PEG: Polyethylene glycol
CT: clotting time	PGG ₂ : prostaglandin G ₂
E: fucogalactan	PGH ₂ : prostaglandin H ₂
ECM: extracellular matrix	PGI ₂ : prostacyclin
EPS: sulfated esculin:	PRP: platelet-rich plasma
5-HT: 5-hydroxytryptamine	PT: prothrombin time
FV: factor V	SBDD: structure-based drug design
FVII: factor VII	SK: streptokinase
FXa: factor Xa	TFG: thrombelastography
FXII: factor XII	TF: tissue factor
FVIII: factor VIII	TO: trehalose octasulfate
GpIb: glycoprotein Ib	TT: thrombin time
GPIIb-IIIa: glycoprotein IIb-IIIa	t-PA: tissues-type plasminogen activator
HAS: human serum albumin	TXA ₂ : thromboxane A ₂
HS: heparan sulfate	TXB ₂ : thromboxane B ₂
sH/HS: shrimp heads/heparan sulfate	UK: urokinase
EC: endothelial cells	vWF: von Willebrand factor
	WB: wheat bran

1. Introduction

Thrombus, a pathologic product of a solid mass (blood clot), can lead to myocardial infarction, ischemic cerebral infarction and other cardio-cerebrovascular diseases. From the 1990s to the present, prevalent cases suffering cardiovascular diseases increase year by year, nearly doubled from 271 million in 1990 to 523 million in 2019, and the number of deaths from 12.1 million in 1990 to 18.6 million in 2019, which account for global mortality and disability (Fig. 1) [1]. In this grim situation, efficiently suppressing thrombosis and thromboembolism can reduce these incidences [2]. Especially since December 2019, the pandemic COVID-19 has broken out across the globe, causing incalculable harm to human health and the socio-economic development. Medical research institutions around the world have been working to develop vaccines in response to the situation, in which the AstraZeneca COVID vaccine, developed in UK, is one of the world's first approved vaccines, but was recently suspended in many countries because of the unpredictable emergence of severe thrombus, and has been banned in Denmark entirely in April 2021. Therefore, developing inexpensive, safer and effective antithrombotic drugs has become a top priority for pharmaceutical researchers, and comprehending the conditions and mechanisms of thrombosis is necessary for drug discovery.

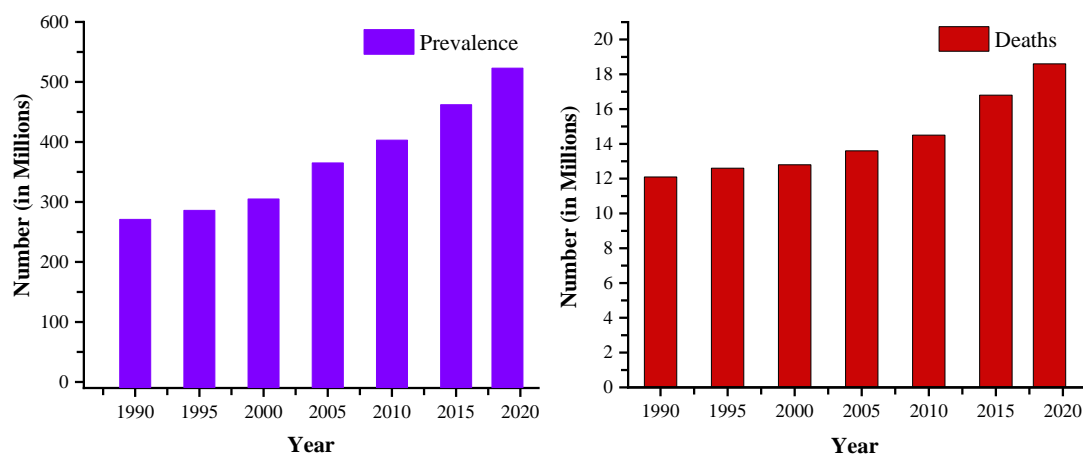


Fig. 1. Total numbers of cardiovascular diseases in global from 1990 to 2019.

Blood has coagulation system and the anticoagulation system (fibrinolysis system) at the same time. In a physiological state, the coagulation factors in the blood

are continuously and limited activated, then produce thrombin and form fibrin in trace amounts, which are deposited in the intima of the cardiovascular system, whereas are dissolved by the active fibrinolytic system immediately. The coagulation system and the anticoagulation system (fibrinolysis system) are dynamically balanced, ensuring both the potential coagulability and the fluid property of the blood. However, when some factors induce the clot emerging, the above dynamic balance is upset, thereby triggering the clotting process which includes platelet aggregation, blood coagulation factor activation and sequential cascade to result in the generation of thrombin and a fibrin clot [3]. The coagulation of flowing blood caused by the activation of platelets and the activation of coagulation factors lead to thrombus formation. Endothelial injury, abnormal blood flow status and hypercoagulability are three major factors predisposing to thrombosis, in which cardiovascular intima injury as the most important and dominant condition, frequently occurring in the heart or in the arterial circulation, is responsible for most cardiovascular and cerebrovascular diseases.

Blood vessel has an inner layer (endothelial cells and subcutaneous basement membrane), a middle layer (smooth muscle cells and their stroma) and an outer layer (fibroblasts and their stroma). Endothelial cell is not just a simple lining of blood vessel, many studies have confirmed that it can secrete a variety of active substances, and possesses anticoagulant and procoagulant functions, but mainly anticoagulant normally. Intact endothelial cells can secrete prostacyclin (PGI₂) and nitric oxide (NO) to inhibit platelet aggregation and dilate blood vessels to resist thrombosis, and thrombin regulate protein on the surface of the endothelial cells could combine with thrombin so as to activate protein C (APC), which would degrade coagulation factor V (FV) and factor VIII (FVIII) to play anticoagulation effect under the assistance of protein S. In addition, tissue plasminogen activator (t-PA) released by endothelial cells can convert plasminogen into plasmin which degrades the water-insoluble fibrin into water-soluble degraded fragments [4]. However, once vascular injury occurs, the underlying basement membrane, termed subendothelial extracellular matrix (ECM) would be exposed, and activate platelets and coagulation factor XII (FXII), initiating the endogenous coagulation process. Simultaneously, injured endothelial cells release

tissue factor (TF) to activate coagulation factor VII (FVII), initiating the exogenous coagulation process. Blood coagulation is initiated by endogenous or exogenous pathways, resulting in coagulation cascades proposed in the early 1960s. In this theory, coagulation is a process that a series of coagulation factors are successively activated by enzymes. Upon the activation of a current coagulation factor, it can cause the activation of the next factor, and then many other coagulation factors would be activated in a certain order, step by step amplification, and finally produce thrombin which can convert fibrinogen into insoluble fibrin to form a large polymerized fibrin net, and then intertwine with platelets and blood cells together to form the thrombus (Fig.2). Moreover, impaired endothelial cells also release plasminogen activator inhibitors (PAI-1) to inhibit fibrinolytic activity and contribute to thrombus stabilization.

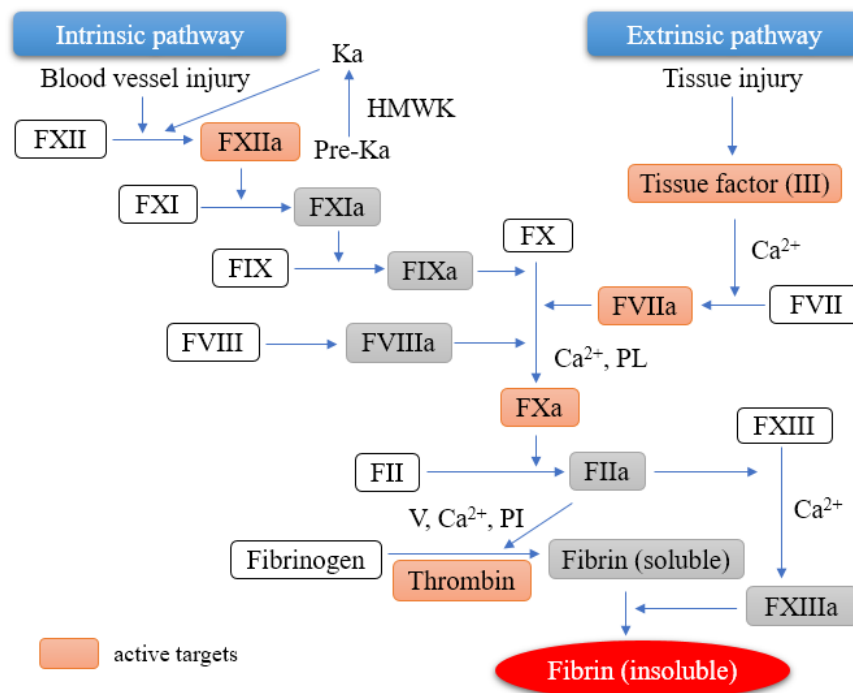


Fig. 2. The cascade mechanism of blood coagulation and the molecular targets.

Activation of platelets plays a central role in triggering the clotting process, and undergo three sequential reactions in the blood clotting (Fig. 3). (1) Adhesion: Platelets adhere to ECM and are activated by vW factor (vWF) which binds the integrin and glycoprotein Ib (GpIb) on the surface of platelets to the underlying basement. (2) Release reaction: Upon activation, platelets release granular products

including fibronectin, ADP, calcium ion (Ca^{2+}), histamine and 5-hydroxytryptamine (5-HT), in which Ca^{2+} is involved in the process of coagulation cascade, and ADP is a powerful mediator of platelet-platelet adhesion. (3) Aggregation: Under the action of thromboxane A_2 (TXA_2) produced by Ca^{2+} , ADP and platelets, platelets in the blood stream continuously adhere to each other and release more ADP and TXA_2 at the same time, which stimulates more and more platelets to form platelet aggregates. The platelet aggregates are reversible in the initial stage of formation, but as the exogenous coagulation process is activated, thrombin is generated and binds to the receptors on platelets to make platelet aggregates further bigger and shrink to become an irreversible platelet mass. In this process, thrombin can convert fibrinogen into insoluble fibrin to form a large polymerized fibrin net, which intertwined with platelets, a few erythrocytes and leukocytes together to be the starting point for thrombus formation. As a crucial component, thrombin is often used as clinical therapeutic target. That is to say, platelet activation, aggregation and fibrin deposition mediated by thrombin lead to thrombosis[5]. Under the synergistic action of continuously produced thrombin, ADP and TXA_2 , platelets in the bloodstream are constantly activated and adhere to the platelet thrombus, causing its increase in size. Blocked by the existing thrombus, blood flow swirls downstream of the clot, forming new mounds of platelets, and so forth, the thrombus gets bigger and bigger, resulting in a series of serious diseases.

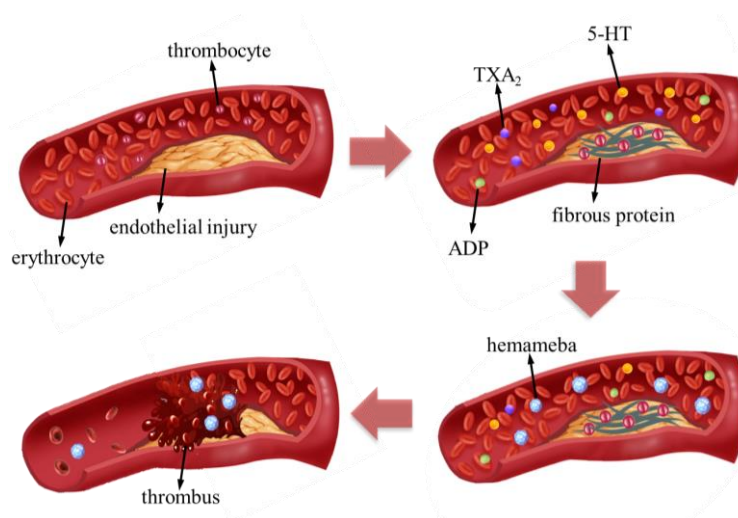


Fig. 3. Process of thrombosis formation.

2. Three categories of approved antithrombotic drugs

As described above, endothelial cells can regulate the balance between anti-thrombotic activity (anticoagulant, antiplatelet and fibrinolytic effects), and prothrombotic activity (platelet, procoagulant and antifibrinolytic effects). In view of these properties, approved antithrombotic drugs have been designed and divided into three categories: anticoagulants, platelet inhibitors and fibrinolytics.

By interfering with the body's physiological clotting process, anticoagulants in clinical application can prevent thrombus formation and limit further development of the pre-existing clots. Thrombin is produced by a series of activated coagulation factors through the initiation of endogenous and exogenous pathways, most factors of which such as FIIa, FVIIa, FIX and FX have serine protein kinase activities. Heparin, a sulfated polysaccharide, is an anticoagulant agent widely used in clinic for treating the patients with venous and arterial thrombotic disorders [6,7], it activate antithrombin III (AT III), leading to the arginine active center of antithrombin combining with serine of many coagulation factors to realize their inactivation (Fig. 4). Heparin or low molecular weight heparin such as enoxaparin, can prolong blood coagulation time with strong and swift action both in vivo and vitro, however they are not orally available and come with the most occurring bleeding side effect owing to no selectivity as well [8]. Fondaparinux (Arixtra®), a synthetic pentasaccharide derivative of heparin approved in 2001 by FDA, can selectively inhibit FXa and thereby has little bleeding risk and more effectivity compared with enoxaparin [9]. Coumarin (2H-chromen-2-one), a natural product from traditional medicine, has a wide range of pharmacological activities and its anticoagulant activity plays a significant role in thrombosis [10]. Warfarin, dicoumarol and acenocoumarol can be used as oral anticoagulants by antagonizing vitamin K, which as an essential cofactor of carboxylase can help carboxylase to carboxylate and activate the precursor of coagulation factors. It is noteworthy in clinic that these coumarins can only prevent the formation of the coagulation factor precursor but have no effect on the formed coagulation factor, which means, they would work only after the formed coagulation

factors are depleted, so they have slow onset of effects and easily lead to bleeding side effects. In addition, thrombin inhibitors like argatroban and hirudin hold important applications in the medical praxis. As a derivative of arginine, argatroban does not affect the process of thrombin formation, but can directly occupy the thrombin active-site to inhibit proteolytic action of thrombin, resulting in the inability of fibrinogen to be converted into fibrin [11]. Hirudin, a polypeptide compound, is the strongest specific inhibitor of thrombin to date. Nowadays, hirudin is often used to be a template to design hirudin variants as potential antithrombotic drugs, whereas, it is restricted in clinical practice owing to the narrow therapeutic window, rapid clearance and risk of bleeding, especially along with other antithrombotic agents [12].

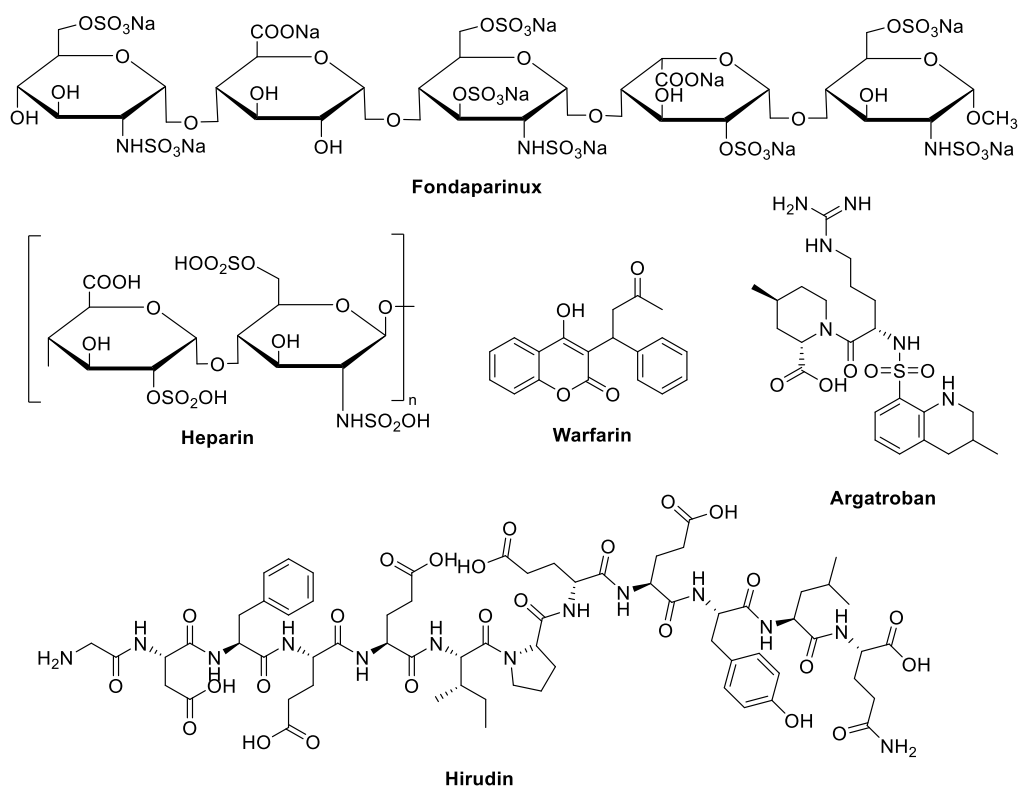


Fig. 4. Chemical structures of common anticoagulants.

Antiplatelet drugs, just as their name implies, can prevent the thrombus formation by inhibiting platelet adhesion, aggregation and release to treat cardiac or cerebral ischemic diseases and peripheral blood thrombotic diseases. In the light of different mechanism of action, three types of antiplatelet drugs including the agents that inhibit platelet metabolism, block ADP-induced platelet activation and platelet membrane glycoprotein (GP) IIb-IIIa receptor blockers are presented here (Fig. 5). In

the first class, TXA₂ plays an important role in clotting formation, some percentage of cardiovascular morbidity and mortality can be attributed to TXA₂ overdose [13]. Phospholipase A₂ stimulates platelet membrane phospholipids to release arachidonic acid (AA), which can be converted into the prostaglandin G₂ (PGG₂) and prostaglandin H₂ (PGH₂) via cyclooxygenase (COX) pathway and the both are further biosynthesized into TXA₂, one of the most potent vasoconstrictors and platelet aggregators, by TXA₂ synthetase [14]. Thus, this class of agents that suppress on PLA₂, COX and TXA₂ will directly or indirectly lead to the reduction of TXA₂ synthesis and inhibition of platelet metabolism, in which COX is the most frequently used as a therapeutic target. As representative drugs in this class, acetyl salicylic acid, named as aspirin, can inactivate COX by acetylating it to perform antithrombotic effect; Ridogrel is a selective TXA₂ synthase inhibitor; Epoprostenol, a PGI₂ congener, has opposing biological effects to that of TXA₂; Dipyridamole, a strong vasodilator, can be used in combination with anticoagulant coumarins to treat thromboembolic diseases. In the second class, Ticlopidine as the representative can selectively interfere with platelet activation mediated by ADP. In the third class, upon activation of platelets, GPIIb-IIIa receptor on platelet exposes new binding site and has a high-affinity binding ability to ligands like fibrinogen, von Willebrand factor (vWF) and endothelium-induced factor and leads to the formation of platelet aggregates [15]. Accordingly, GPIIb-IIIa receptor blockers can prevent platelet from binding to these ligands and inhibit platelet aggregation at maximum extent [16].

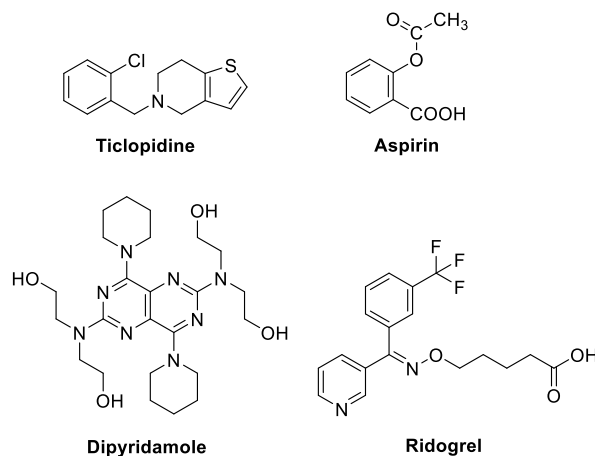


Fig. 5. Chemical structures of common antiplatelet drugs.

Fibrinolytics is also called thrombolytics because it can convert plasminogen into plasmin which rapidly hydrolyzes fibrin and fibrinogen and causes thrombolysis. The first generation of fibrinolytics was represented by streptokinase (SK) and urokinase (UK). SK, a non-enzymatic single-stranded protein combine with plasminogen to form a complex which facilitates the transformation of plasminogen into plasmin that hydrolyzes fibrin in thrombus to cause thrombolysis. UK, a glycoprotein extracted from urine, can directly transforms plasminogen into plasmin to achieve the procedure of dissolving thrombus. Although SK and UK possess potently thrombolytic activity, they have low specificity and high risk of bleeding. t-PA as a representative in second generation of fibrinolytics has a good selectivity and dispense with the drawbacks of the first generation [17]. Alteplase, a kind of glycoprotein containing 526 amino acids, is the most commonly used thrombolytic drug and the only one approved by the FDA for the treatment of acute ischemic stroke [18].

In recent years, the discovery of novel antithrombotic molecules has attracted considerable attention in medicinal chemistry field. With regard to diverse medicinal mechanism, these new molecules are also divided into anticoagulants, platelet inhibitors and fibrinolytics.

3. New antithrombotic molecules

3.1 Anticoagulants

3.1.1 Coumarin-substituted derivatives

Amin et al. [19] designed and synthesized a battery of 6-heterocycle substituted coumarin derivatives **1**, **2a**, **2b**, **3a-3e** for the anticoagulant effects using warfarin as reference substance (Fig. 6). In a mouse thrombus model, most of compounds showed similar clotting time (CT) and prothrombin time (PT) values compared to their precursor 6-aminocoumarin. The most active compound oxadiazole **1** (CT: 17.70 ± 0.88 s, PT: 42.30 ± 2.32 s) showed the familiar PT value of warfarin (CT: 23.20 ± 1.25 s, PT: 42.30 ± 1.61 s). All derivatives relied on inhibition for FXa, one of the most attractive targets of oral anticoagulants, resulting in a decrease of thrombin to achieve the anticoagulant effect with less risk of bleeding [20,21].

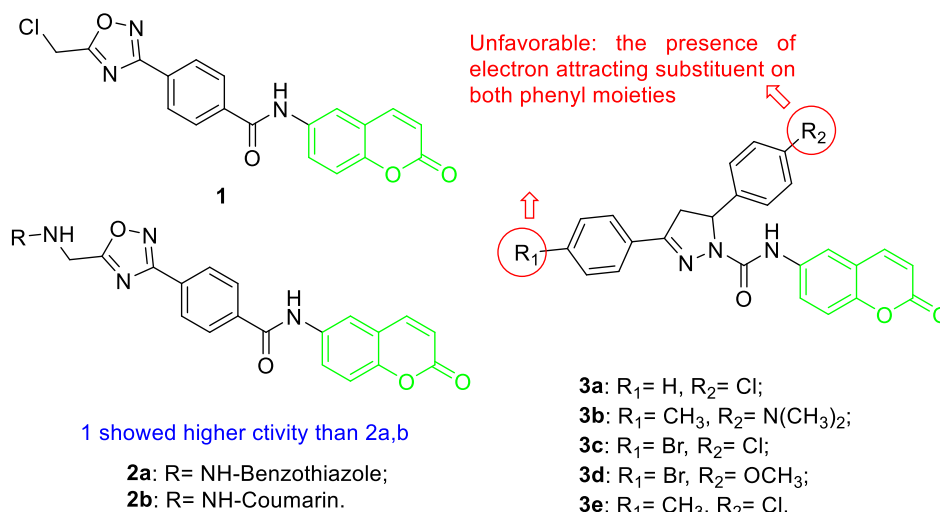


Fig.6. Chemical structures of 6-heterocycle substituted coumarin derivatives 1-3.

In 2018, enlightened by the natural coumarin derivative esculin with anticoagulant activity, Ahmad et al. [22] synthesized a sulfated esculin **4** and evaluated its anticoagulant ability (Fig. 7). In vitro clotting times, PT, activated partial thromboplastin time (APTT) and thrombin time (TT) exhibited to be delayed in the presence of **4** compared with esculin. Additionally, the antithrombotic effect of **4** in vivo showed significant decreases in thrombotic rat model, suggesting that **4** can be used as a promising potential agent with dual anticoagulant and antithrombotic effects.

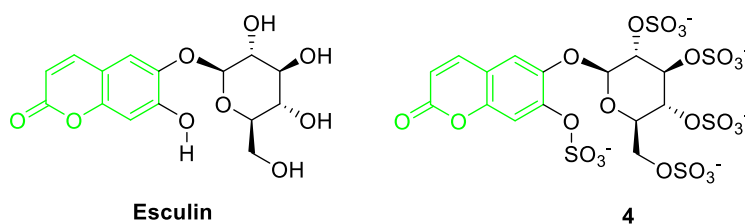


Fig.7. Chemical structures of esculin and sulfated esculin-4.

Bang et al. [23] synthesized a series of coumarin conjugates as indirect anticoagulants, the anticoagulant activity of these compounds were evaluated by thrombin progenitor time in mice (Fig. 8). All synthetic compounds except **5c** (PT: 8.44 ± 2.35 s) had greater PTs than the reference drug warfarin (PT: 7.97 ± 1.93 s), and compounds **5a** (PT: 10.88 ± 0.56 s) and **5b** (PT: 13.10 ± 3.56 s) had the best anticoagulant effects.

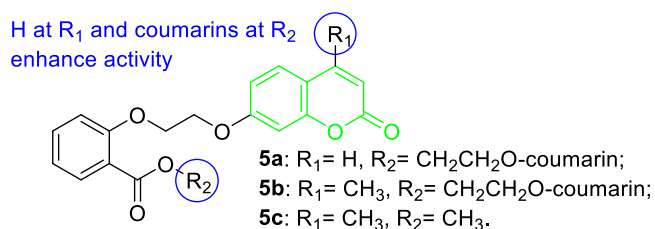


Fig.8. Chemical structures of coumarin conjugates **5**.

Kasperkiewicz et al. [24] synthesized coumarin derivatives for the development of new anticoagulant potential drugs with higher efficacy, longer half-life, minor side effects and safer administration in comparison to warfarin (Fig. 9). The experimental data (bioactivity, drug-likeness and blind docking) explored by computational examinations demonstrated that all the compounds had a better safety profile than warfarin, interestingly, compound **6b** ($n = 1.64$, n is the number of ligands binding to the protein) with two methoxy groups in 3- and 4-position in benzene ring was found to have two binding sites with human serum albumin (HSA), while the others ($n < 1$) were only one binding site, which can be attributed to smaller steric hindrance of **6b** than that of other derivatives.

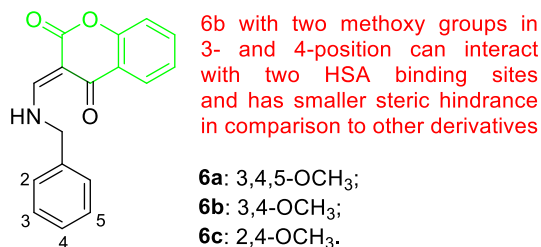


Fig.9. Chemical structures of coumarin derivatives **6**.

To investigate the activity of quercetin metabolites in cardiovascular disease, Shi et al. [25] synthesized quercetin derivatives (Fig. 10). The thrombus model of male white rabbit showed that in the monomethylated series, the most active compounds were **7a** (TT: 19.40 ± 1.15 s, APTT: 7.60 ± 0.64 s, PT: 49.20 ± 5.51 s) and **7b** (TT: 23.00 ± 1.17 s, APTT: 6.80 ± 0.38 s, PT: 72.60 ± 5.07 s), and in the dimethylated series, the optimal one was **7c** (TT: 21.80 ± 0.85 s, APTT: 6.90 ± 0.48 s, 67.60 ± 6.95 s) whereas **7d** (TT: 9.90 ± 1.05 s, APTT: 5.80 ± 0.36 s, 58.70 ± 3.10 s) showed no better anti-thrombic activity than quercetin, although it prolonged APTT.

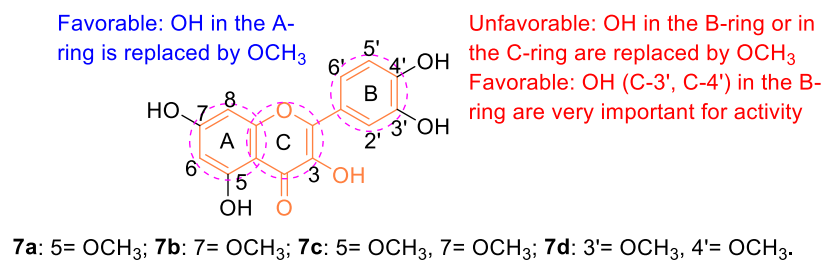
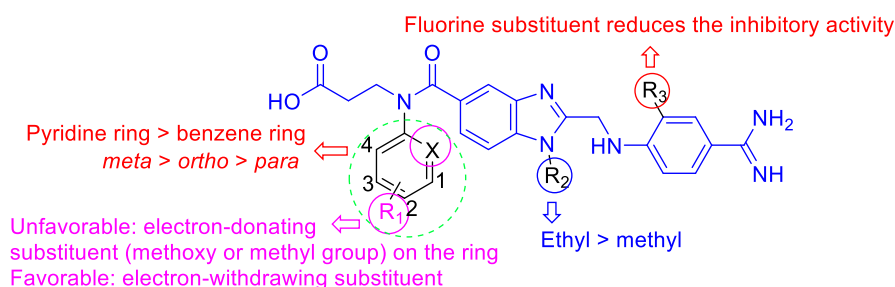


Fig.10. Chemical structures of quercetin derivatives **7**.

3.1.2 Dabigatran derivatives

Li et al. [26] synthesized fluorinated dabigatran derivatives **8a-8d**, and prodrugs **9, 10** of compounds **8a** and **8c**. **8a-8d** with IC₅₀ values of 3.49, 3.71, 3.23 and 3.39 nM respectively, are comparable to the reference drug dabigatran (IC₅₀: 2.61 ± 0.84 nM) (Fig. 11). Compound **10**, the prodrug of compound **8a**, had a strong inhibitory effect with an inhibitory rate of 73 ± 6%, similar to dabigatran 76 ± 2% in the rat thrombus model. Moreover, they synthesized a series of fluorinated 2,5-substituted-1-ethyl-1H-benzimidazole derivatives [27]. All the 9 derivatives with effective activity can be used as direct thrombin inhibitors (Fig. 12). Among them, the most potent compound **11h** (IC₅₀: 3.39 ± 0.10 nM) displayed a good inhibition rate by chromogenic assays comparable to dabigatran (IC₅₀: 2.61 ± 0.84 nM) and most of them showed moderate to good thrombin inhibitory activity. The inhibitory activities of compounds **11a** (IC₅₀: 5.63 ± 0.10 nM), **11b** (IC₅₀: 7.26 ± 0.91 nM), **11d** (IC₅₀: 8.40 ± 0.09 nM) and **11e** (IC₅₀: 4.92 ± 0.29 nM) were better than that of argatroban (IC₅₀: 9.46 ± 0.92 nM). According to the molecular docking model, the compound **11h** had strong binding affinity with amino acid residues of thrombin active site to play its activity.



8a: X= N, R₁= H, R₂= CH₃, R₃= F; **8b:** X= C, R₁= 2-F, R₂= CH₂CH₃, R₃= F;
8c: X= C, R₁= 2-F, R₂= CH₃, R₃= H; **8d:** X= N, R₁= H, R₂= CH₂CH₃, R₃= F.

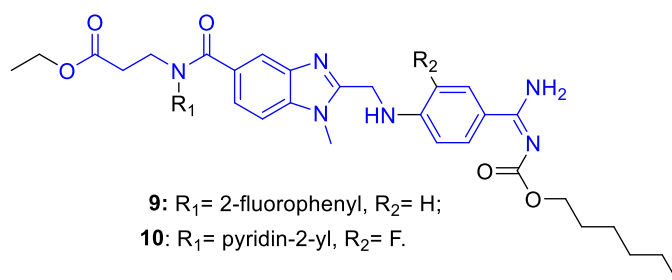
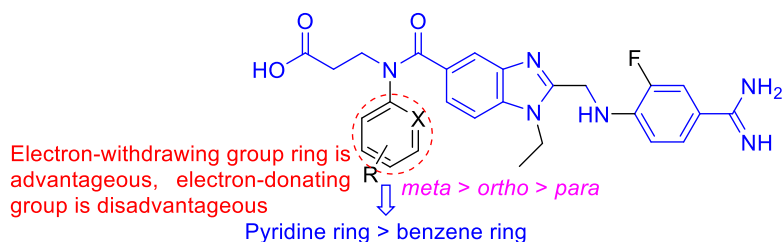


Fig.11. Chemical structures of fluorinated dabigatran derivatives **8-10**.

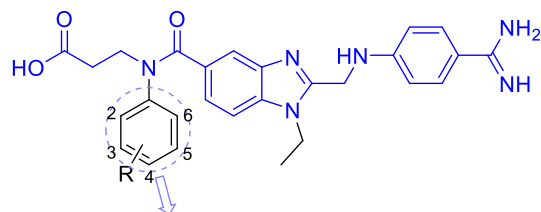
Substituent group at the *para* position of the amino group on a benzene ring or a heterocyclic ring decreases the activity



11a: X= C, R= H; **11b:** X= C, R= 2-CH₃; **11c:** X= C, R= 2-OCH₃;
11d: X= C, R= 3-OCH₃; **11e:** X= C, R= 3-F; **11f:** X= C, R= 4-OCH₃;
11g: X= C, R= 4-Cl; **11h:** X= N, R= H; **11i:** X= N, R= 5-CH₃.

Fig.12. Chemical structures of fluorinated 2,5-substituted 1-ethyl-1H-benzimidazole derivatives **11**.

The inhibitory effects of compounds **12a-12f** on thrombin were stronger than that of argatroban (IC₅₀: 9.46 ± 0.92 nM). In combination with the benefits of anti-thrombin drugs and fluorine atoms in the treatment process, Chen et al. [28] designed and synthesized 9 new fluoride anti-thrombin derivatives by introducing fluorine into thrombin inhibitors (Fig. 13). Compound **12a** (IC₅₀: 3.52 ± 0.32 nM) is the most effective inhibitor of thrombosis in chromogenic assay. Molecular docking studies have found that compound **12a** interacts with Asp36, His102, Trp53 and Tyr101 to form hydrogen bonds with thrombin active site.



Favourable: the F group at the *ortho* position;
the FF group at the 3,5-position
Unfavourable: the CF₃ group in the *ortho*,
meta, and *para* position; the F group in the
meta and *para*

12a: R= 2-F; **12d:** R= 3,4-F₂; **12g:** R= 3-CF₃;
12b: R= 3-F; **12e:** R= 3,5-F₂; **12h:** R= 4-CF₃;
12c: R= 4-F; **12f:** R= 2-CF₃; **12i:** R= 4-OCF₃.

Fig.13. Chemical structures of fluoride anti-thrombin derivatives **12**.

Based on bioisosteric and scaffold hopping principles, Chen et al. [29] synthesized two dabigatran mimics (**13a** and **14**), and in vitro antithrombotic efficacy study showed that both **13a** (IC₅₀: 9.20 nM) and **14** (IC₅₀: 7.48 nM) had excellent anticoagulant effect (Fig. 14). To extend oral application, a total of 21 ester and carbamate derivatives of the two mimics mentioned above were developed by replacing benzimidazole moiety of dabigatran with tricyclic fused scaffold and evaluated for the anticoagulant activity. **13b** (IC₅₀: 0.64 ± 0.11 μM) had the most excellent property by conducting a series of experiments (including evaluation in vivo antithrombotic efficacy, mice tail bleeding test, rabbit thrombin-induced platelet aggregation assay and molecular modeling study), indicating that **13b** was safer than dabigatran (IC₅₀: 0.34 ± 0.06 μM) in bleeding risk, with a shorter bleeding time at the same dose in mice-bleeding test.

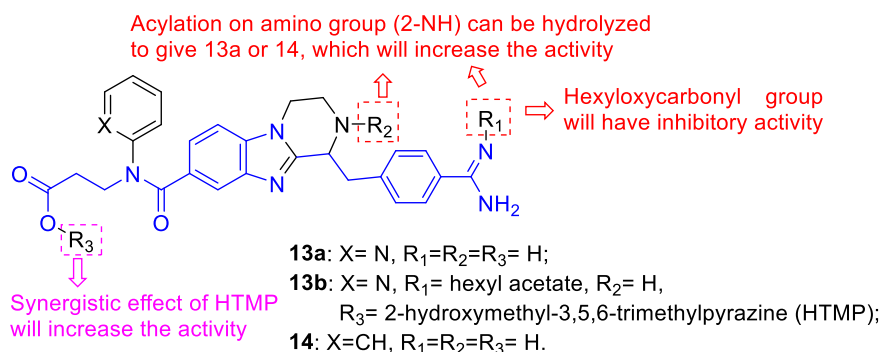


Fig.14. Chemical structures of dabigatran mimics **13-14**.

Ren et al. [30] designed, synthesized and evaluated a class of *N*-ethyl dabigatran derivatives **15a-15p** for inhibition of thrombin activity and most showed similar

inhibitory activity compared to dabigatran (IC_{50} : 1.20 nM) (Fig. 15). Among them, the most potent compound **15p** (IC_{50} : 0.96 nM) displayed a good inhibition rate of (85.35 ± 0.72) % at concentration of 1 mg/mL for arteriovenous thrombosis based on blood clotting in rat model. Meanwhile, compounds **15a-15e**, **15h** and **15i-15n** showed the similar inhibition activities as dabigatran, compound **15f** showed weaker activity than the others. The SAR revealed that N substituted on the phenyl ring (X position) could improve the activity and introduction of the *o*-position methyl was more beneficial to the activity compared with *p*-position methyl. According to molecular docking study, compound **15p** could form hydrogen bonds with Asp189 to bind the target thrombin protein strongly.

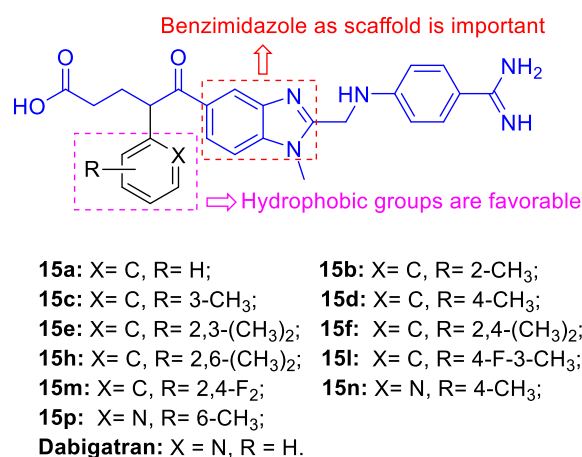


Fig.15. Chemical structures of *N*-ethyl dabigatran derivatives 15.

3.1.3 Biological polysaccharide derivatives

Inspired from heparin, the widely used drug to treat thrombosis as known [31-33], Patey et al. [34] synthesized a series of heparin derivatives **16a-16h** by systematic modification at the main sites of *O*-sulfation and *N*-sulfation/acetylation (Fig. 16). The anti-FXa activities of these compounds determined by the specific pentasaccharide sequence showed that compound **16a** (IC_{50} : 0.03 μ g/mL) had the best activity and the interference ability of these derivatives to antithrombin III/FXa complex was greatly affected by *N*-sulfate substitution with *N*-acetyl group.

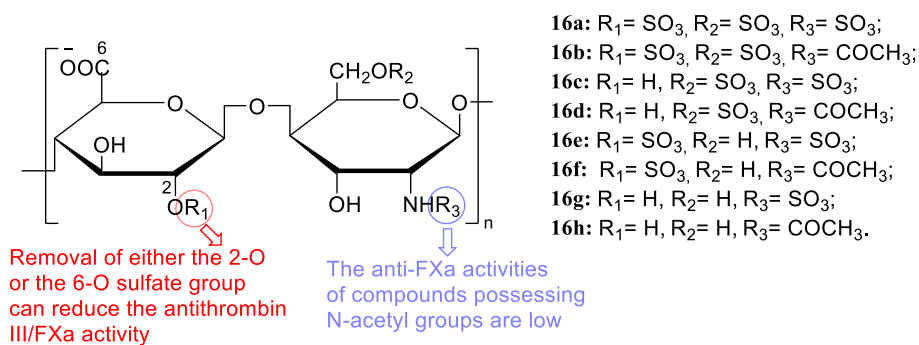


Fig.16. Chemical structures of heparin derivatives **16**.

Román et al. [35] extracted fuco-galactan (E) from *agaricus bisporus*, and got sulfated E after further modification to obtain more polysaccharides with anticoagulant effect. As expected, all sulfated E derivatives had different anticoagulant activities and the best one had great response on APTT but showed lesser activity compared with heparin, indicating that sulfated E maybe inhibit the intrinsic pathway rather than extrinsic pathway of blood coagulation.

Marine brown algae, a rich source of sulfated polysaccharide, can be used as a potential anticoagulant. Based on the property of brown algae, Rocha et al. [36] reported that sulfated galactofucan, a new sulfated polysaccharide from brown algae (Fig. 17), had no anticoagulant effect in vitro, whereas showed potent antithrombotic activity with maximum 8 h after administration in animal models of venous thrombosis, much longer than heparin. It was proposed that the sulfated galactofucan can facilitate the synthesis of antithrombotic heparan sulfate by endothelial cell to display the effect indirectly, indicating that it can be used as a potentially antithrombotic agent.

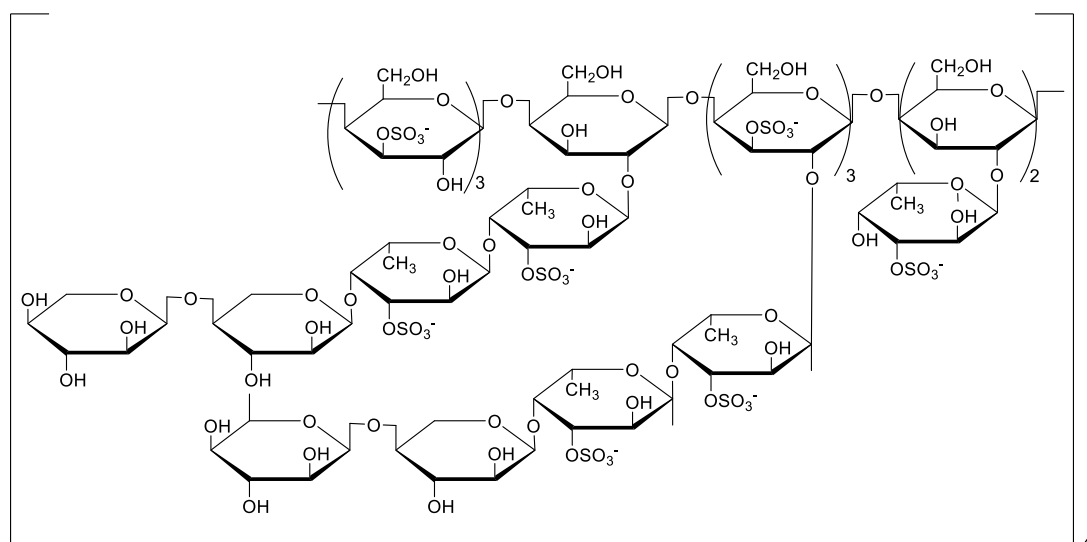


Fig.17. Chemical structures of the proposed sulfated galactofucan.

Brito et al. [37] found that a hybrid heparin/heparan sulfate-like compound from *Litopenaeus vannamei* shrimp heads (sH/HS) showed strong anticoagulant and anti-FXa activity with slight bleeding effect. Given strong anticoagulant and anti-FXa activity related to anti-cancer, they further studied the inhibitory potential of sH/HS on thrombin activity and its effect on tumorigenesis. In the thrombin inhibition test, sH/HS showed much greater inhibitory effect than heparin (90.70%, 55.30%, respectively) at 0.50 $\mu\text{g}/\text{mL}$ concentration, while from 1.0 $\mu\text{g}/\text{mL}$ concentration, both polysaccharides showed similar inhibitory effect, reaching about 95%. Furthermore, the effects of different concentrations of sH/HS or heparin on the synthesis of antithrombotic heparan sulfate in endothelial cells (EC) of rabbit aorta confluence showed that sH/HS compounds were effective as heparin. In addition, sH/HS inhibited the migration of B16F10 cells without affecting normal cellular activity, and showed a 90% decrease in tumor growth. In the case of cancer related thromboembolic diseases, these findings make sH/HS a good focus that inhibits tumor progression and development, as well as prevents coagulopathy.

β -D-xylopyranosides were reported to have antithrombotic activity in previous literature [38], and the SAR indicated that substitution of sulfur for the sugar-ring oxygen and the glycosidic oxygen atoms could lead to an upsurge of activity. 4-cyanophenyl glycoside of 1,5-dithio- β -D-xylopyranose (beciparcil) had fairly well

activity, however, the severe side effects prevented it from being a potential clinical drug. Inspired by the antithrombotic activity of beciparcil, Bozo et al. [39] synthesized the corresponding 2-deoxy analog **17** in order to optimize beciparcil's structure to reduce the side effects, with beciparcil as a control to evaluate the antithrombotic activity of **17** in rat model (Fig. 18). The result displayed that **17** (ED₅₀: 7.0 mg/kg) was much safer than beciparcil (ED₅₀: 25.0 mg/kg), suggesting that **17** could be a promising antithrombotic agent.

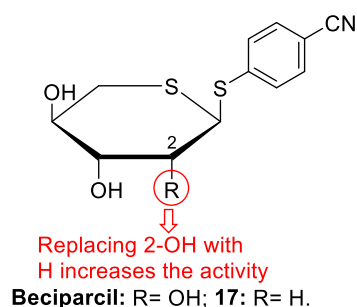


Fig.18. Chemical structures of beciparcil and **17**.

Xiong et al. [40] selectively synthesized two water-soluble chitosan (WSC) derivatives (Fig. 19), *N*-succinyl-chitosan (**18**) and *N,O*-succinyl-chitosan(**19**). A rabbit model of thrombus was employed to study their antithrombotic, hemolytic and anticoagulant properties which were expressed by some pharmacological parameters including APTT, PT and TT to display their anticoagulant performance in normal rabbit plasma. At a concentration of 3 mg/mL, compared to chitosan as control group (APTT = 33.80 s, PT = 12.00 s, TT = 18.10 s), **18** (APTT = 48.20 s, PT = 12.00 s, TT = 18.20 s) and **19** (APTT = 125.30 s, PT = 12.10 s, TT = 18.60 s) could prolong APTT, while no obvious increase of PT and TT was observed, suggesting that they may inhibit intrinsic rather than extrinsic coagulation pathway.

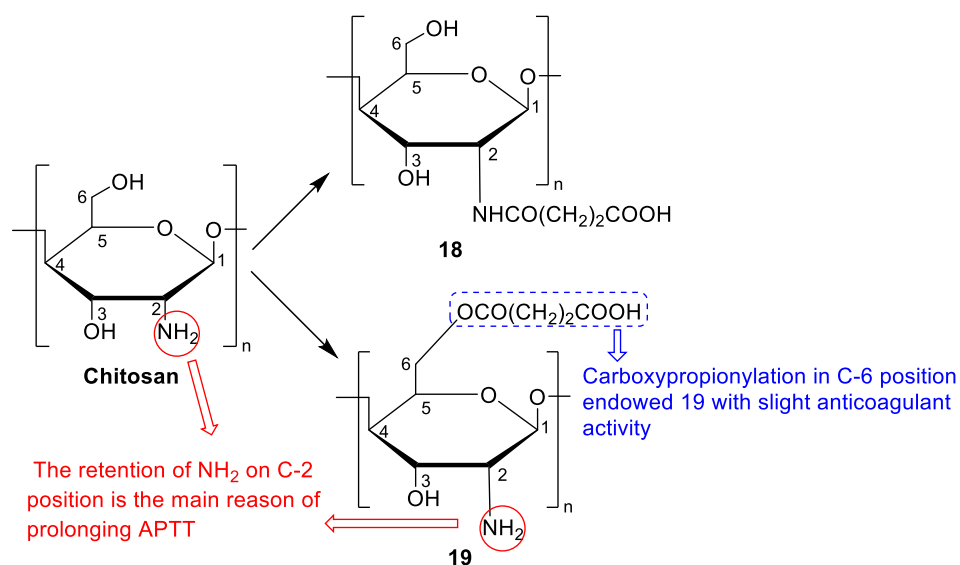


Fig.19. Chemical structures of WSC derivatives 18-19.

Bozo' et al. [41] synthesized thioglycosides **20 α** , **20 β** , **21 α** , **21 β** and their D-isomers **22 α** , **22 β** , **23 α** , **23 β** (Fig. 20). The antithrombotic activity of these compounds tested in rats model showed that **20 α** (ED₅₀: 15 mg/kg) had slight more potency than beciparcil (ED₅₀: 25 mg/kg), and all the other glycosides (ED₅₀: 1~5 mg/kg) were much stronger compared to beciparcil.

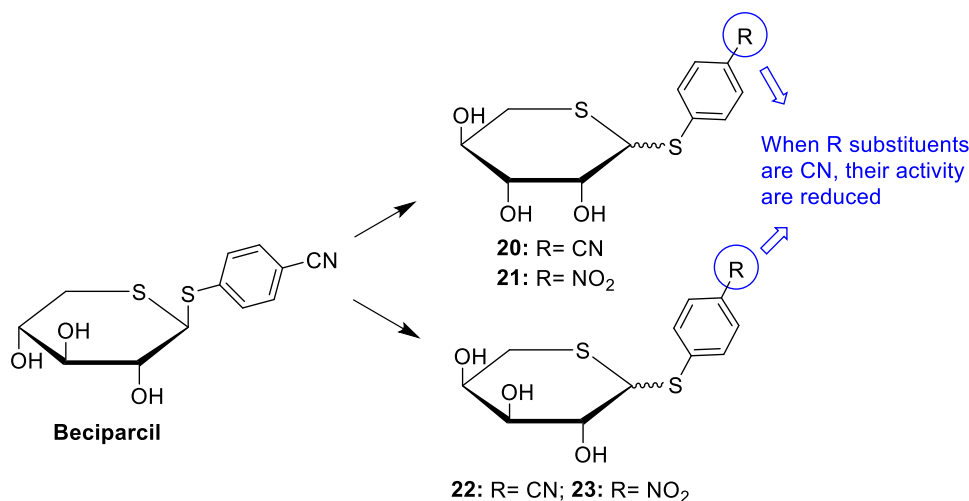


Fig.20. Chemical structures of thioglycosides 20-23.

3.1.4 Derivatives from traditional Chinese medicine

Based on good thrombin inhibitory activity of glycyrrhizin and its aglycone glycyrrhetic acid, Paula et al. [42] designed and synthesized 3 glycyrrhizin derivatives **24a-24c** (Fig. 21). Compound **24b** was a phthalate derivative of glycyrrhizin with better thrombin inhibition (IC₅₀: 114.40 ± 1.30 μM) compared to the

saponin (IC_{50} : $235.70 \pm 1.40 \mu M$) in human plasma and it can prolong TT (118.30 ± 8.00 s). According to molecular docking, it was speculated that **24b** could restrain the hydrolysis of substrate **S2238** (H-D-Phe-Pip-Arg-*p*-nitroanilide), a synthetic peptide specially designed for the catalytic site of thrombin, to exhibit anticoagulant activity.

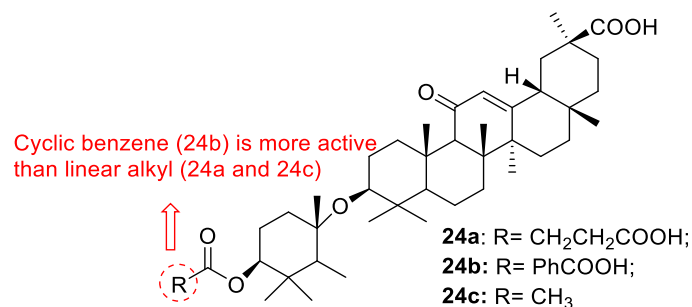


Fig.21. Chemical structures of glycyrrhizin derivatives **24**.

Isosteviol, a hydrolysate derived from natural stevioside, is a tetracyclic diterpene containing beyerane skeleton, with unique advantages such as low toxicity, low cost and excellent biological characteristics. Chen et al. [43] have discussed that compound **25** contained the tetracyclic diterpene skeleton with significant anticoagulant and antiplatelet activity, and developed a series of isosteviol derivatives (**25a-25r**) containing effectively bioactive oxime ether (Fig. 22). Compound **25k** exhibited excellent selectivity for a panel of serine proteases and the best activity against FXa ($K_i = 0.015$ mM), as well as showed potent anticoagulant activity ($p < 0.01$) and antiplatelet activity ($p < 0.01$) according to the PT and APTT analysis in vitro and the rat ADP-induced platelet aggregation test in vitro (clopidogrel as the positive control). Furthermore, the in vivo and in vitro antithrombotic activity experiments on rats displayed compound **25k** had prominent antithrombotic activity (in vitro $p < 0.01$, in vivo $p < 0.0001$), with rivaroxaban and clopidogrel as positive controls, indicating that compound **25k** can inhibit the factors of various stages of thrombus formation, and could be developed as a promising antithrombotic drug.

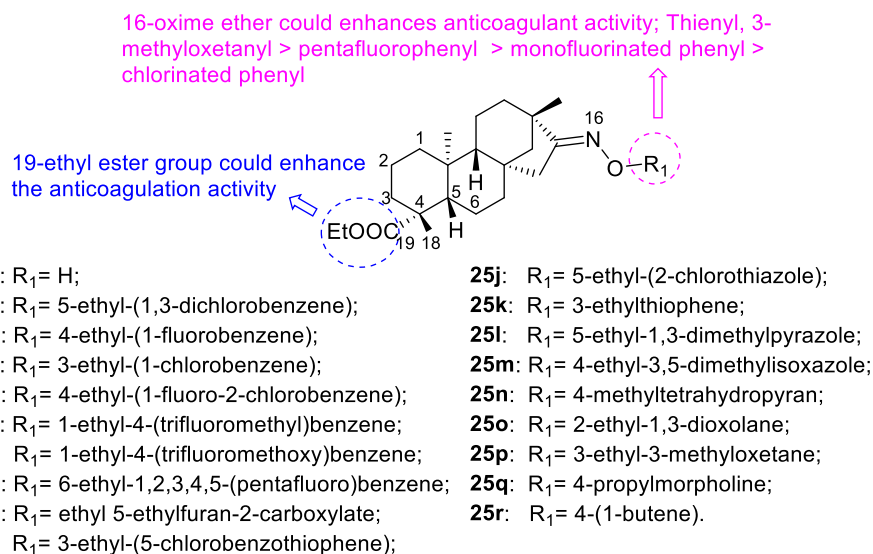


Fig.22. Chemical structures of isosteviol derivatives 25.

3.1.5 Other derivatives

Some previous works showed that *N*-[(7-amidino-2-naphthyl) methyl] aniline derivatives were effective FXa inhibitors [44,45]. **YM-60828**, one of the derivatives, had a good antithrombotic effect and its L-shaped conformation at the active site of FXa is considered to play an important role in the inhibitory activity. Hirayama et al. [46] synthesized the **YM-60828** derivatives by cyclizing on the side chain of amino acetic acid and the central benzene ring, and discussed their activities on FXa (Fig. 23). These compounds showed great anticoagulant activities in vitro. **26a** (IC₅₀: 2.10 nM), **26b** (IC₅₀: 2.80 nM) and **26c** (IC₅₀: 2.50 nM) showed better FXa inhibitory and anticoagulant activity compared to **YM-60828** (IC₅₀: 6.00 nM). Furthermore, **26c** (PT₁ = 4.00 h, PT₂ = 8.30 h) showed the best anticoagulant activity and effectively prolonged PT in mice and squirrel monkey thrombus model, compared with **YM-60828** (PT₁ = 2.60 h, PT₂ = 4.30 h). Later, they obtained several effective oral FXa inhibitors by optimizing the central aniline nitrogen side chain of 7-amino-2-naphthylaniline [47], including a series of 7-amidino-2-naphthoanilide (Fig. 24). Among these compounds, **27a-27j** showed favorable efficiency in inhibition for FXa, and **27h** (IC₅₀: 3.90 nM) was the most effective, possessing a strongest FXa inhibitory activity in squirrel monkey model at the dose of 3 mg/kg.

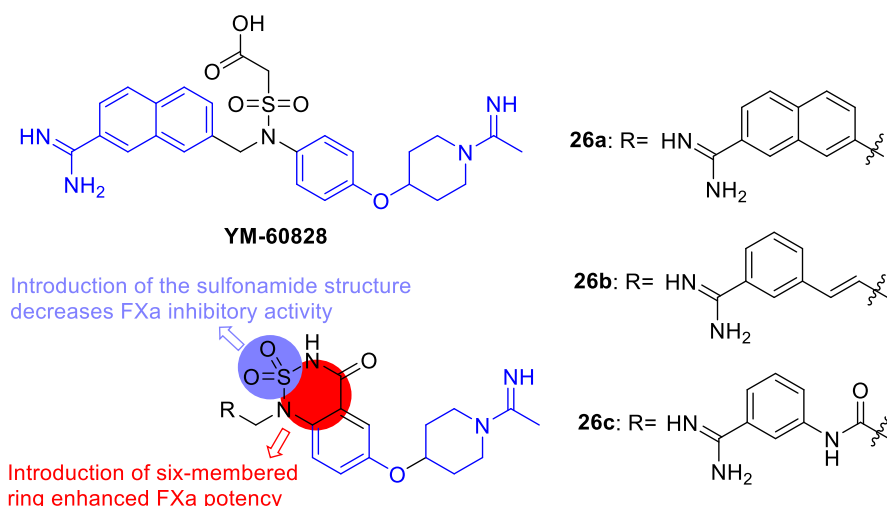


Fig. 23. Chemical structures of **YM-60828** and **26**.

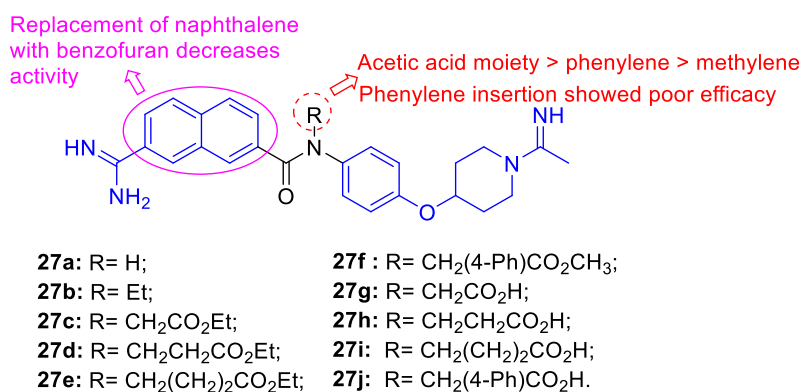


Fig.24. Chemical structures of oral FXa inhibitors **27**.

Based on the previously reported compound **28** [48], Lu et al. [49] synthesized a series of novel non-amino acid thrombin inhibitors guanidine analogues **29-33** (Fig. 25). The in vitro selectivity towards thrombin and the crystal structures of these compounds were investigated, and it was proved that compound **29** had a remarkable selectivity ($>10^3 \times$) and inhibition for thrombin, whereas even the concentration was reached to 200 μM , it had no inhibitory effect on other enzymes such as urokinase, FXa, plasmin, chymotrypsin and trypsin.

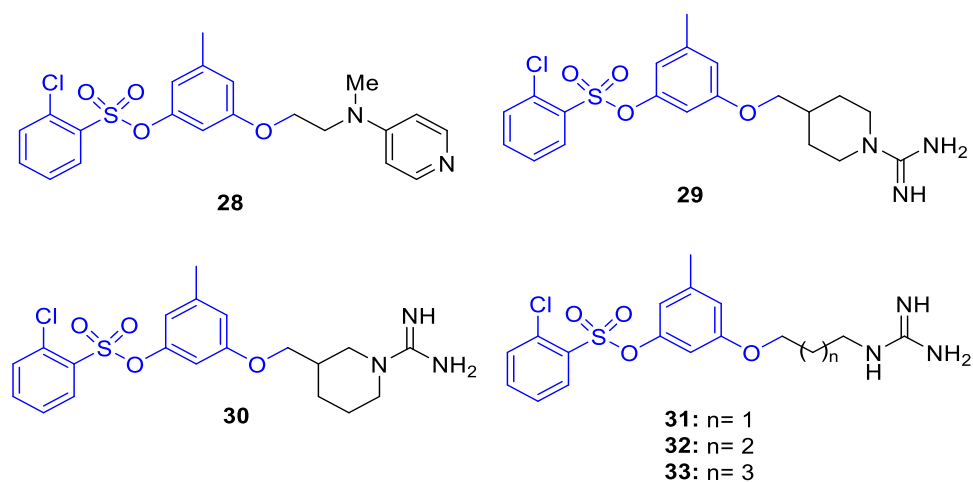


Fig.25. Chemical structures of thrombin inhibitors **28-33**.

Based on the strategy of combining amidine derivatives and their prodrugs, Ishihara et al. [50] synthesized a series of *N*-benzenesulfonylpiperidine derivatives to excavate potent and orally active FXa inhibitors (Fig. 26). Compound **34b** exhibited potent inhibitory activity against FXa (IC_{50} : 13 nM) and remarkable selectivity over thrombin (> 7000-fold) within the coagulation cascade. The prodrug **34a** from the parent **34b**, without activity in vitro, could show an anticoagulant effect via oral administration by biological conversion into its corresponding active ingredients.

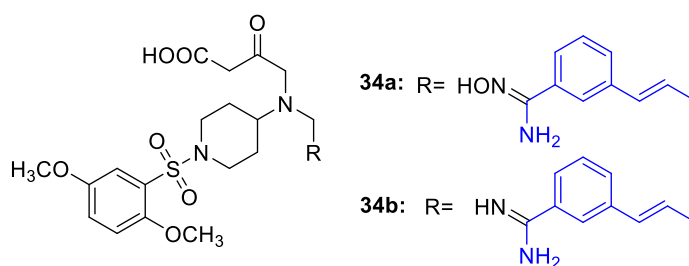


Fig.26. Chemical structures of *N*-benzenesulfonylpiperidine derivatives **34**.

Encouraged by the combination of thrombin inhibitors and fibrinogen IIb/IIIa antagonist with more benefits in previous work [51,52], Ilic et al. [53] synthesized 1,4-benzodioxine enantiomeric compounds by incorporation of anticoagulant and antiplatelet aggregation activity into the same molecule to be dual antithrombotic drugs, and investigated the effects of chirality and regional heterogeneity on these potential dual antithrombotic compounds (Fig. 27). Compound **35d** was the most effective thrombin inhibitor ($K_{i(thr)}$: $0.078 \pm 0.005 \mu M$), and compound **36b** had the best inhibitory activity at both targets of thrombin and fibrinogen GPIIb/IIIa $K_{i(thr)}$:

$1.67 \pm 0.27 \mu\text{M}$, $\text{IC}_{50}(\text{GPIIb/IIIa}): 0.66 \pm 0.26 \mu\text{M}$).

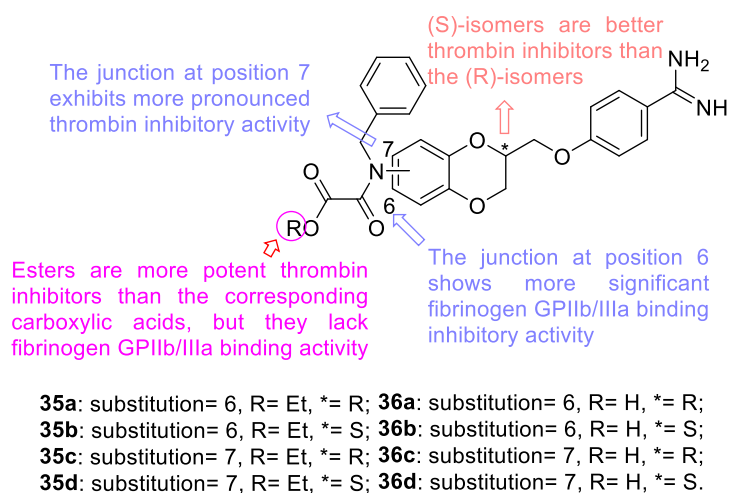


Fig.27. Chemical structures of 1,4-benzodioxine enantiomeric compounds **35** and **36**.

Liu et al. [54] designed and synthesized a series of new 3,4 - diaminobenzoyl derivatives with highly selective inhibition on FXa (Fig. 28). The anticoagulant activity of these compounds was determined by PT in rabbit plasma in vitro. Compounds **37a** (PT = 10.20 ± 2.00 s), **37b** (PT = 8.80 ± 1.70 s) and **37c** (PT = 9.50 ± 0.40 s) displayed significant anticoagulant activity, and among them, **37a** (PT = 10.20 ± 2.00 s) showed greater inhibition than apixaban (PT = 10.00 ± 1.50 s).

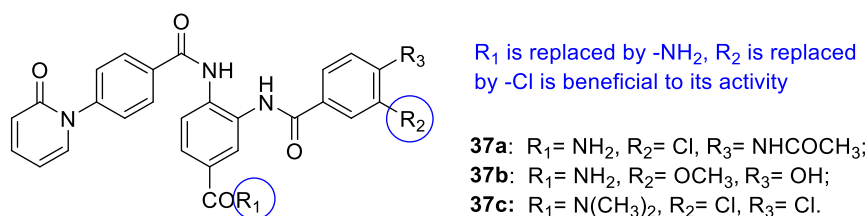


Fig.28. Chemical structures of 3,4 - diaminobenzoyl derivatives **37**.

Amide bond (-CONH) was exploited by Tarasov et al. [55] for linking different moiety to form a series of novel FXa inhibitors like $\text{P}_1\text{-(CONH)-P}_2\text{-(CONH)-P}_3$ (Fig. 29). Compound **38**, with doubled prothrombin time (PT $\times 2$) of $0.08 \pm 0.02 \mu\text{M}$, was the most active amide inhibitor of FXa (IC_{50} : 0.50 ± 0.08 nM). The half-life and activity of **38** were superior to other compounds in rabbit and mouse models of thrombus.

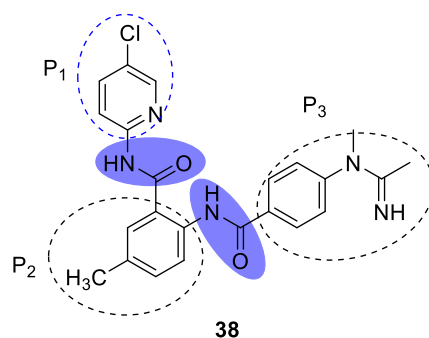


Fig.29. Chemical structures of FXa inhibitors **38**.

In the process of coagulation, prothrombin is converted into active thrombin after combination with thrombin factors, and the generated thrombin can accelerate the combination in turn. One strategy of inhibitor design relies on the powerful action between the positively charged amidinium ions on the thrombin inhibitors and the side chains of Asp189 on the serine protease (FXa is a kind of serine protease). Wiley et al. [56] used the 1,2-dibenzamidobenzene derivative **39** (a competitive inhibitor of FXa with $K_i = 1.00 \mu\text{M}$ [57]) as the research object to explore the attachment of amidine substituents of compounds **40** to the inhibitor **39** in order to enhance the interaction with Asp189 (Fig. 30). According to FXa affinity, molecular simulation, evaluation of anticoagulation and antithrombotic activity, FXa affinity and selectivity can be significantly increased by the addition of an amidine to the side chain of dibenzamidobenzene derivatives. These compounds can execute effective anticoagulant and antithrombotic activity in vivo.

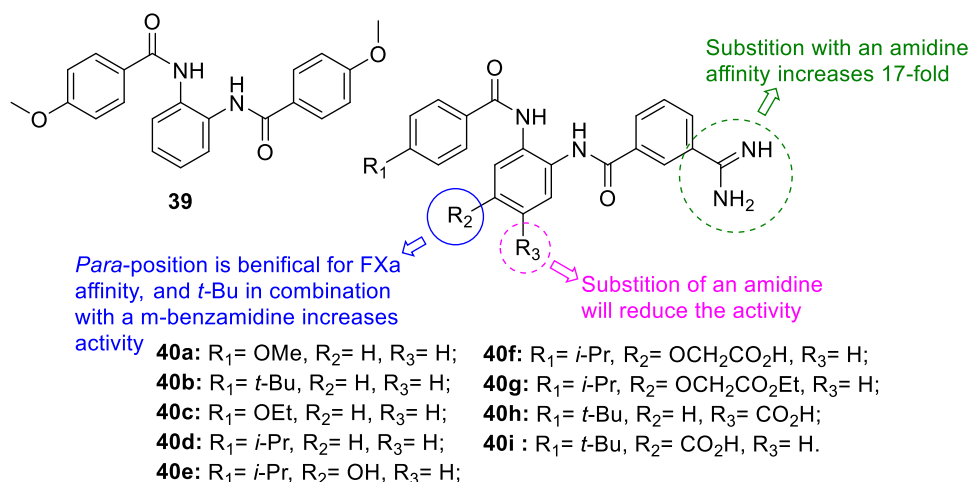


Fig.30. Chemical structures of 1,2-dibenzamidobenzene derivative **39** and **40**.

The formation of TF/VIIa complex by the combination of tissue factor (TF) with

serine protease factor VIIa (FVIIa) in the blood induces the formation of fibrin clots and leads to thrombus formation, and inhibition for the TF/VIIa complex could not only block the formation of thrombosis but also effectively reduce the side effect of bleeding. Analysis for the crystal structure of tripeptide- α -ketothiazole (effectively inhibiting serine protease) combined with TF/VIIa showed that 3-pyridylphenylalanine played an important role in the selectivity for the TF/VIIa complex [58], so they used benzyl-pyrazinone as the active group to synthesize a series of compounds **41a-41c** [59] via structure-based drug design (SBDD) and 5-step PASP library (Fig. 31). These compounds were tested in TF/VIIa enzyme, FXa, thrombin, and trypsin respectively, it was found that **41c** had the best effect, with an IC_{50} value of 16 nM and more 6,250 times selectivity for TF/VIIa than FXa and thrombin.

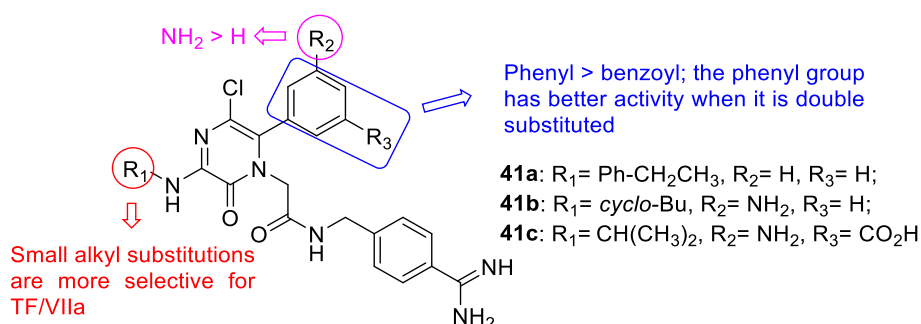


Fig.31. Chemical structures of benzyl-pyrazinone derivatives **41**.

In 2015, Xing et al. [60] synthesized the compounds **42** and **43** as well as their analogues **42a-42g**, **43a-43e**, respectively (Fig. 32). Betrixaban was used as a positive control to evaluate the inhibitory activity of all compounds on FXa in vitro. Most compounds had significant activity against FXa (IC_{50} : 0.023-1.12 μ M), in which **43b** was the most effective one with IC_{50} value of 23.0 nM, and the selectivity of compound **43b** ($2 \times PT = 8.70 \mu$ M) for thrombin was comparable to the reference betrixaban ($2 \times PT = 2.40 \mu$ M). In 2017, using compound **44** with prominent inhibitory activity against FXa as a template, they designed and synthesized a battery of 2,3-dihydroquinazolin-4(1H)-one derivatives **45a-45h** for developing novel scaffold of FXa inhibitors [61] (Fig. 33). Compound **45e** was the most promising agent with potent FXa inhibitory activity (IC_{50} : 21.00 ± 6.00 nM) along with a reduction in thrombus weight, showing similar effectiveness and safety to betrixaban

in arteriovenous shunt (AV-SHUNT) model of rats. In addition, **45e** had a remarkable selectivity against thrombin with the IC₅₀ value of 67.0 μM.

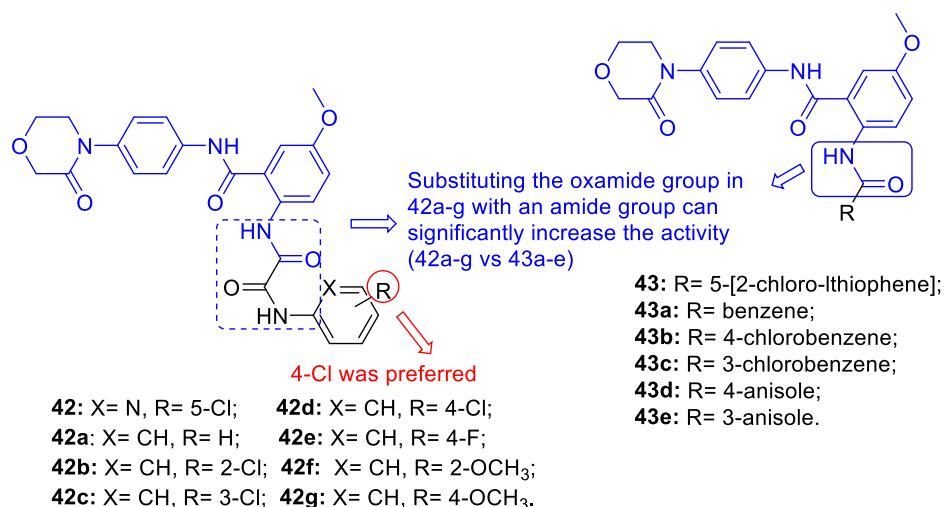


Fig.32. Chemical structures of the compounds **42-43**.

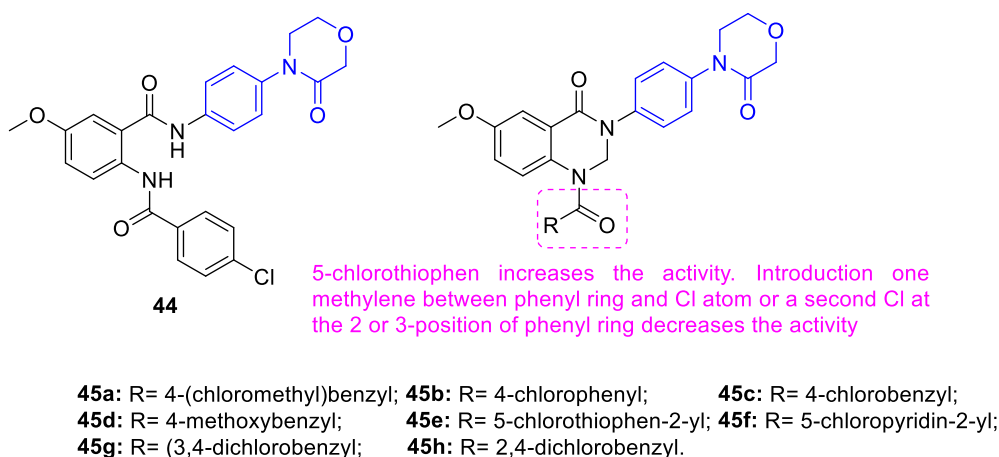


Fig.33. Chemical structures of 2,3-dihydroquinazolin-4(1H)-one derivatives **44-45**.

Although different FXa inhibitors possess various structures, most of them are constituted of three components consisting of a main scaffold and two hydrophobic moieties. With Betrixaban as a template, a series of anthranilamide derivatives **46a-46j** were developed as novel FXa inhibitors [62], in which **46g** had the best activity (IC₅₀: 3.50 nM) both in vitro and in vivo AV-SHUNT thrombosis model of rats (Fig. 34). In addition, **46g** was more effective than betrixaban, with higher thrombus weight reduction and more safety at the same dose of 1 mg/kg and 5 mg/kg.

4-chlorophenyl is the best surrogate to increase the activity
 4-methyl phenyl or 4-ethynylphenyl also showed a potent activity

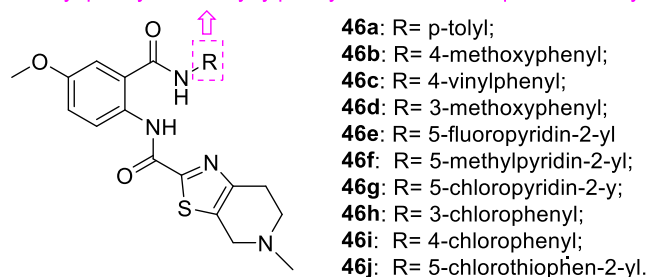


Fig.34. Chemical structures of anthranilamide derivatives **46**.

Based on a dataset containing a total of 38 new tricyclic fused oxazolidinones which have been reported by Yang [63], Xu et al. [64] conducted a series of studies using computational methods including molecular dynamics (MD) simulation, 3D-QSAR, molecular docking and Topomer CoMFA, and studied the conformation or orientation of [6,6,5] tricyclic fused oxazolidinones and offered constructive information for the design of novel FXa inhibitors (Fig. 35). Among all the compounds, compound **47a** had the highest bioactivity with actual pIC₅₀ (-logIC₅₀) value of 8.60, and could be employed as the template molecule to illustrate the MD simulations of other compounds.

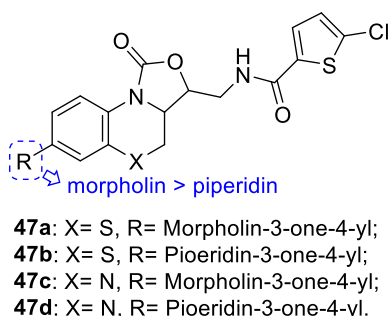


Fig.35. Chemical structures of [6,6,5] tricyclic fused oxazolidinones derivatives **47**.

In view of the catalysis of FXa in thrombin generation, Yang et al. [65] synthesized a series of isoxazolo [5,4-d] pyrimidin-4(5H)-one derivatives, subjected to FXa inhibitors with less bleeding complications [66] (Fig. 36). Compound **48g** with acetoxyl group exhibited better FXa inhibitory activity (IC₅₀: 0.013 μM) and higher selectivity than other compounds and showed almost the same activity with rivaroxaban's (98% versus 100%) at the concentration of 0.10 μM. Moreover, **48g** also displayed good anticoagulant potency in human plasma (2 × PT = 2.12 μM).

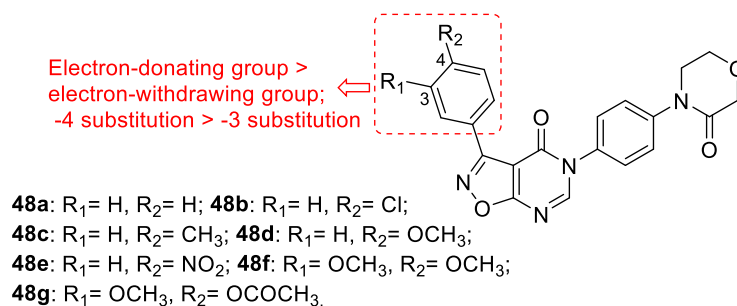


Fig.36. Chemical structures of isoxazolo [5,4-d] pyrimidin-4(5H)-one derivatives **48**.

According to benzothiazole guanidine fragment with weak thrombin inhibitory activity, Michael et al. [67] designed and synthesized a series of new type of benzothiazole guanidine compounds **49a-49g** with low pK_a values (Fig. 37). Among them, **49a-49c** displayed different degrees of ability to bind to thrombin, while **49d-49g** displayed an effect on thrombin. In the thrombin activity assay, **49b** was the most active one, **49a** showed slight inhibition at high concentrations, and **49c** was proved to be more effective than **49a**, the unsubstituted one at -N.

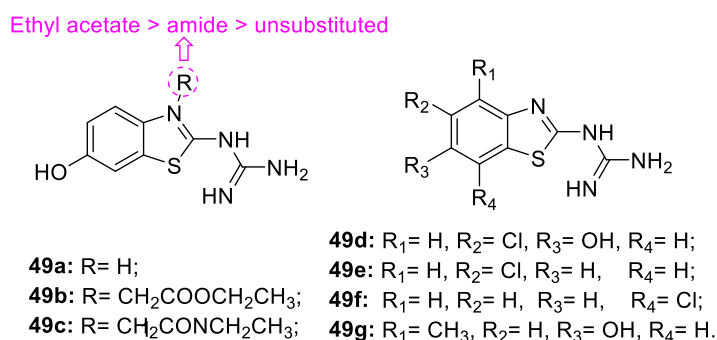


Fig.37. Chemical structures of Benzothiazole guanidine fragments **49**.

Taking into account that the non-amidine inhibitor can undergo a bioconversion into the corresponding conjugate to enhance anticoagulant activity in vivo and in vitro by increasing hydrophilicity, Ishihara et al. [68] exploited antithrombotic compound **50a** as a template to synthesize thrombin inhibitors with phenol as the functional group which could bind to the potential substrate to form glucuronic glycoside conjugates with high anticoagulation effect after biological transformation by oral administration (Fig. 38). The in vitro selective activity screening of human thrombin and trypsin showed that compound **50b** with phenolic hydroxyl group at the 3-position (IC₅₀: 0.0040 μM) and compound **50c**, its corresponding bioconverted glucuronide (IC₅₀: 0.0049 μM) had the similar activity to **50a** (IC₅₀: 0.0058 μM) in

inhibition on thrombin factor FXa. The intravenous bolus injection experiment and the anticoagulant activity test in cynomolgus monkeys showed, compared with compounds **50a** and **50b**, the anticoagulant activity of **50c** (the doses of 0.10 mg/kg) was significantly improved, and its inhibitory activity was 10 times higher than that of compound **50a** (the doses of 1.00 mg/kg). Taken together, compound **50b** was a promising oral anticoagulant inhibitor.

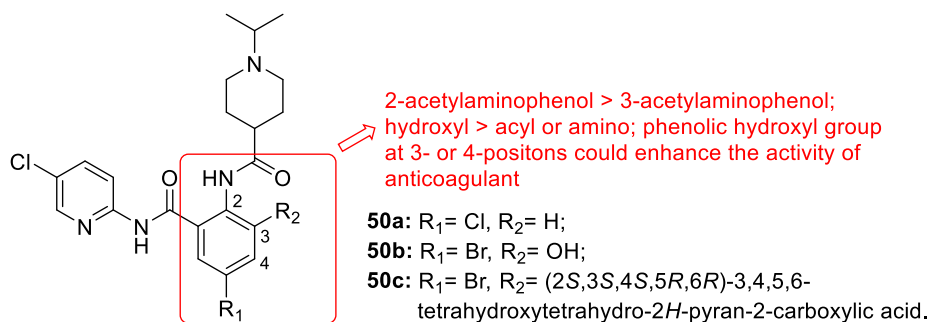


Fig.38. Chemical structures of antithrombotic compound **50**.

FXII as a trypsin-like serine protease can promote coagulation through contact activation, and in thrombus caused by medical-instrument-mediated contact, the inhibition for FXII can effectively increase antithrombotic activity without bleeding side effects [69,70]. Bouckaert et al. [71] designed a series of oxygen-containing compounds (-OH or -COOH), using coumarin derivative (serine protease inhibitor) as the framework (Fig. 39). Moreover, as a result of lack for antithrombotic models in vivo, the FXIIa hybrid model was conducted by combining the advantages of the FXIIa homology model and the FXII protein crystal structure. The activity experiment of single concentration screening at 50 mM on FXIIa showed that **51a-51e** had inhibitory activity and compound **51a** had the best activity (IC₅₀: 5.00 μM). Further, calculations of the binding energy about these compounds to ligands showed that **51a** was the most stable compound attributed to the minimum energy. Subsequently, molecular docking displayed that targeting the H1 pocket (unique to FXIIa) was the reason for the selectivity of compound **51a**, according to superimpose the crystal structures of proteases (thrombin, FVIIa, and FXa) and the complex of FXIIa mixed model.

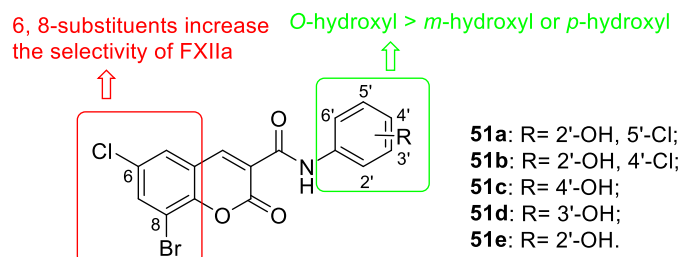


Fig.39. Chemical structures of coumarin derivative **51**.

Since the C-7 carbamate side chain of vorapaxar is a metabolic site, Chelliah et al. [72] cyclized the carbamate in the form of a spiral ring to obtain a range of C7-spirocyclic analogues of vorapaxar (**Fig. 40**), **52a-52d** (spirocyclic oxazolidinone analogues), then evaluated their activities against PAR-1 receptor by using rat thrombus model. Among those compounds, 2-cyanophenyl analogue **52c** ($K_i = 5.10$ nM) was the most active derivative.

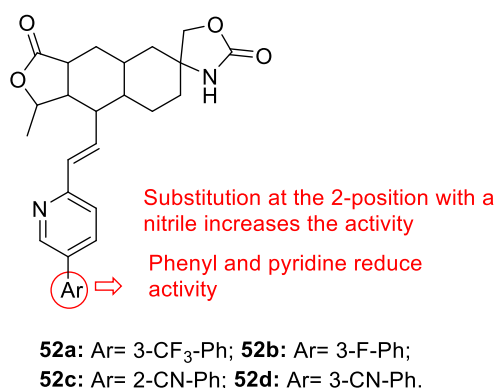


Fig.40. Chemical structures of spirocyclic oxazolidinone analogues **52**.

Lucietto et al. [73] designed and synthesized two cyclic dipeptides **53a** and **53b** (**Fig. 41**), and found that the both had significant inhibitory effects on thrombin, whereas **53b** only showed a 43.30 % reduction at lower concentration (0.025 mM). In addition, **53b** did not distinctly inhibit thrombin-induced platelet aggregation, but **53a** did (IC_{50} : 0.066 mM).

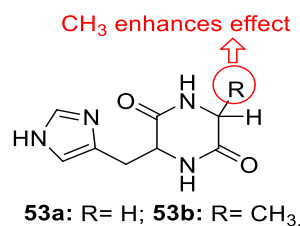


Fig.41. Chemical structures of cyclic dipeptides **53**.

Based on the antithrombosis drugs reported in the literature mentioned above, we summarized their respective targets and structural characteristics, and found that FXa and thrombin as the attractive targets accounted for majority in drug development, shown in Table 1.

Table 1. Anticoagulant compounds and their respective targets

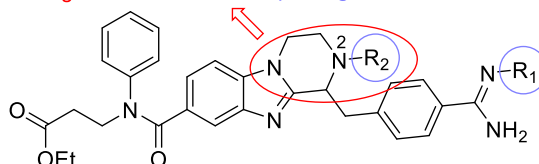
NO.	Compound name	Target	Structural characteristics
1	1~3	FXa	Coumarin derivative
2	4	Thrombin	Coumarin derivative
3	6	HSA	Coumarin derivative
4	7~15	Thrombin	Dabigatran derivative
5	16	FXa	Biological polysaccharide derivatives
	24	Thrombin	glycyrrhizin
7	25~27	FXa	Isosteviol
8	28~33	Thrombin	/
9	34	FXa	amidine
10	35~36	Thrombin, fibrinogen GPIIb/IIIa	1,4-benzodioxine enantiomeric
11	37~40	FXa	/
12	41	TF/VIIa	benzyl-pyrazinone derivative
13	42~48	FXa	/
14	49	Thrombin	benzothiazole guanidine fragment
15	50	FXa	/
16	51	FXIIa	coumarin derivative
17	52	PAR-1	spirocyclic oxazolidinone analogue
18	53	α -glucosidase	cyclic dipeptide

3.2 Platelet inhibitors

Using thrombin inhibitor **54** with a new framework of tetrahydrobenzo [4, 5] imidazole and [1, 2-a] pyrazine as the lead compound, Chen et al. [74] designed and synthesized amino formic acid ester derivatives (Fig. 42). Biological activity test (in

vitro) results showed that all the compounds had certain inhibitory activities on the platelet aggregation induced by thrombin. The inhibitory activity of compound **55b** (IC_{50} : $0.32 \pm 0.10 \mu\text{M}$), **57b** (IC_{50} : $0.57 \pm 0.11 \mu\text{M}$) and **58b** (IC_{50} : $0.47 \pm 0.06 \mu\text{M}$) is comparable to that of dabigatran (IC_{50} : $0.60 \pm 0.05 \mu\text{M}$), whereas compound **56b** (IC_{50} : $0.11 \pm 0.08 \mu\text{M}$) is better. In vitro inhibitory activity of the four precursors (compounds **55b-58b**) was generally better than that of their counterparts in the dual precursors (compounds **55a-58a**). In vivo mouse model of thrombus, it is speculated that these derivatives may be hydrolyzed to produce HTMP (ethanol extraction of Chuanxiong) with antiplatelet aggregation activity.

The 2-NCH₃ derivative of R₁ and R₂ are both substituted for amino formic tetrahydropyrazine ring is weaker acid ester (55b-58b), which is better than just than its corresponding 2-NH derivative replacing amino formic acid ester in R₂ (55a-58a)



- 54**: R₁ = R₂ = H
55a: R₁ = CH₂COOCH₂CH₃, R₂ = H; **55b**: R₁ = R₂ = CH₂COOCH₂CH₃;
56a: R₁ = CH₂COOC(CH₃)₃, R₂ = H; **56b**: R₁ = R₂ = CH₂COOC(CH₃)₃;
57a: R₁ = CH₂COO(CH₂)₄CH₃, R₂ = H; **57b**: R₁ = R₂ = CH₂COO(CH₂)₄CH₃;
58a: R₁ = CH₂COOCH₂-Ph, R₂ = H; **58b**: R₁ = R₂ = CH₂COOCH₂-Ph.

Fig.42. Chemical structures of amino formic acid ester derivatives **54-58**.

Tian et al. [75] synthesized novel 2H-benzo[b][1,4]oxazin-3(4H)-ones (Fig. 43), in which **59a-59g** had good activities for antiplatelet aggregation, and compound **59a** had the strongest inhibitory effect (IC_{50} : $10.14 \mu\text{M}$), but the effectiveness is lower than that of the reference material ticlopidine (IC_{50} : $3.18 \mu\text{M}$) and aspirin (IC_{50} : $6.07 \mu\text{M}$). Based on the SAR, the inhibition of platelet receptor - binding compounds can be ameliorated by bringing down 4 - and 7-position steric hindrance. Moreover, a series of 2H-benzo[b][1,4]-oxazin-3(4H)-ones were synthesized [76] in 2014 based on previous work [75], and aspirin and ticlopidine were used as controls to evaluate the activity of inhibition on ADP-induced platelet-rich plasma in New Zealand rabbits (Fig. 44). As the strongest antiplatelet compound **60** (IC_{50} : $9.20 \mu\text{M}$) is comparable to aspirin (IC_{50} : $7.07 \mu\text{M}$).

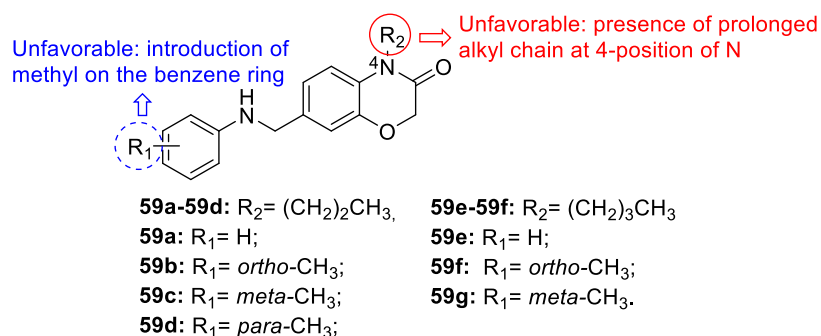


Fig.43. Chemical structures of 2H-benzo[b][1,4] oxazin-3(4H)-one derivatives **59**.

Smaller steric hindrance group in R_1 (ethyl, isopropyl and propyl) > larger group (benzyl)

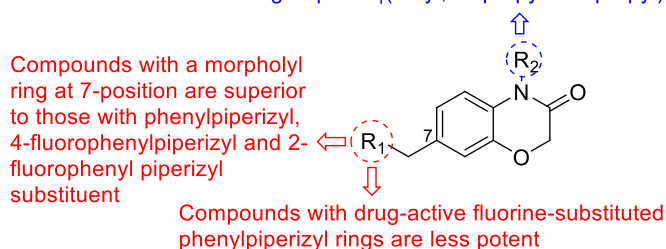


Fig.44. Chemical structures of 2H-benzo[b][1,4]-oxazin-3(4H)-ones **60**.

Taking allicin as the basic structure, Block et al. [77] synthesized the difluoro allicin **61** and its related organosulfur compounds, as well as compared the effects of these fluorinated organosulfur compounds on platelet and coagulation activation by human whole blood thrombelastography (TEG) (Fig. 45). Difluoro allicin **61** (51.0 μM) showed similar effect to that of allicin (62.0 μM , $p < 0.001$) in antiplatelet activity, whereas stronger than that of allicin and other organic sulfur compounds in anticoagulant activity. Fluorine substitution enhanced the electrophilicity and reactivity of these compounds against the biological mercaptan groups, so the substitution of F atom and the presence of $-S=O$ group made compound **61** the most active.

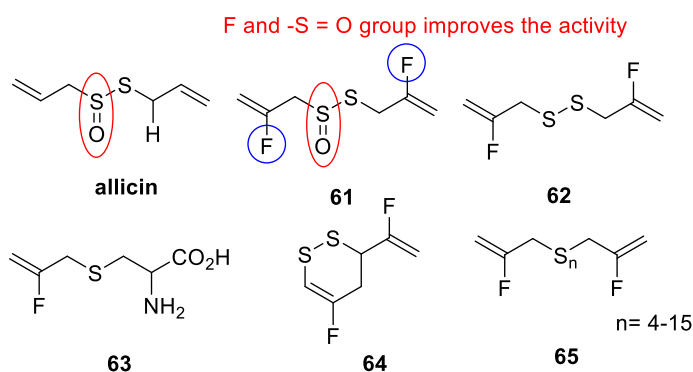
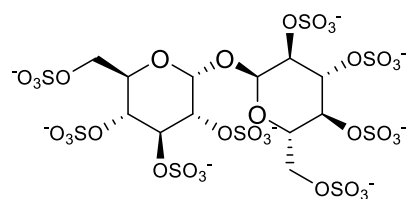


Fig.45. Chemical structures of difluoro allicin and its related organosulfur compounds **61-65**.

Rashid et al. [78] synthesized a novel small sulphated molecule trehalose octasulfate (TO) with anticoagulant and antiplatelet effects (Fig. 46). In vitro and in vivo coagulation tests, TO significantly prolonged the clot time of APTT and PT, as well as showed good anticoagulant property in a dose-dependent manner. When evaluating antiplatelet activity in rat thrombus model, decreased platelet aggregation was observed in blood collected from animals exposed to TO, indicating that TO has dual action mechanism and exhibits antithrombotic property.



Trehalose octasulfate

Fig.46. Chemical structures of sulphated molecule **TO**.

Marrakchi et al. [79] isolated a series of lebetin (L1; 11- to 13-mer) from *Macrovipera lebetina* venom which can inhibit platelet aggregation induced by various agonists (Fig. 47). After purification, **66 α** , **66 β** and **66 γ** were obtained, which contained 13, 12 and 11 amino acid residues respectively. *N*-Terminal truncation of **66 α** was used to synthesize **66 β** and **66 γ** , and their activities were studied. Based on alanine screening, the substitution of residues and the integrity of polypeptide chains affected antiplatelet activity.

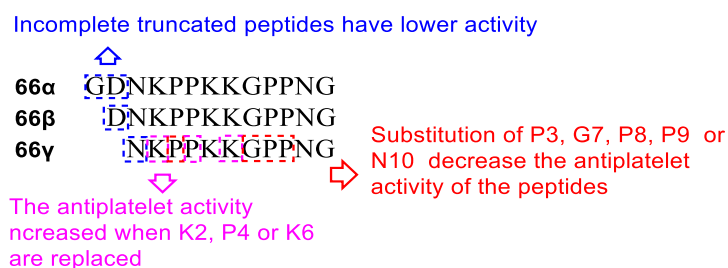


Fig.47. Chemical structures of **66 α** , **66 β** and **66 γ** .

The activation of P2Y₁ receptor could give rise to ADP-mediated platelet aggregation, on the contrary, antagonism of the P2Y₁ receptor can block the aggregation and it is widely used as a validated target for antithrombosis. In 2007, Costanzi et al. [80] employed adenosine 3',5'-diphosphate (A3P5P), a kind of competitive antagonist of human P2Y₁, as a template to synthesize a bunch of A3P5P

derivatives (Fig. 48). *N*-methanocarba derivative **67c** showed the best antithrombotic activity in vivo, with the IC₅₀ of 8.40 nM. The R substituent of compound **67e-67g** (IC₅₀: 6.90 ± 2.50 μM, 1.75 ± 1.10 μM, 0.87 ± 0.18 μM) with cyano group, polar carboxyl group and carboxyl amide at the position 2 respectively had poor tolerance to P2Y₁ receptor. The antagonistic activity of 2-acetylenyl substituted compound **67k** (IC₅₀: 0.093 ± 0.0030 μM) was much higher than that of its corresponding compound **67l** (IC₅₀: 2.40 ± 0.60 μM) with a longer hexynyl chain. Compound **67h** (IC₅₀: 0.048 ± 0.001 μM) with shorter chain length has higher affinity and potency than that of compound **67i** (IC₅₀: 0.45 ± 0.21 μM), **67j** (IC₅₀: 1.87 ± 0.59 μM), **67l** (IC₅₀: 2.40 ± 0.60 μM) with longer chain length. Similarly, less bulky compound **67k** is more liable to bind to the P2Y₁ receptor compared with compound **67m** with phenyl-substituted at the position 2. Compound **68** (IC₅₀: 1.56 ± 0.54 μM), a derivative of **67c** with the phosphate group removed, would result in disappearance of activity, and compound **69a** (IC₅₀: 0.62 ± 0.040 μM) and **69b** (IC₅₀: 0.79 ± 1.60 μM) with halogen substitution both had the general activity.

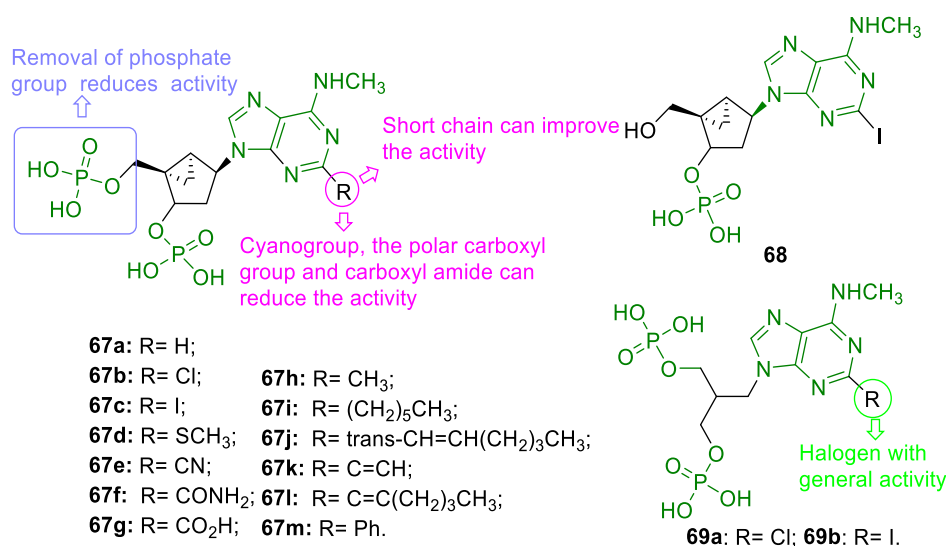


Fig.48. Chemical structures of A3P5P derivatives **67-69**.

Compound **70** synthesized by the predecessor is not highly selective for P2Y₁ receptor ($K_B = 100$ nM) [81], whereas it shows significant affinity for P2X₁ and P2X₃ receptors, with an IC₅₀ value of 1.15 and 12.90 μM, respectively [82]. To develop novel P2Y₁ antagonists as new antithrombotic agents, Raboisson et al. [83] prepared

*N*⁶-dimethyl-1-2'-deoxyadenosine-3',5'-bisphosphate derivatives **71a-71d**, among them, **71d** as an antagonist is 4 times more effective than **70** in the platelet aggregation test (Fig. 49).

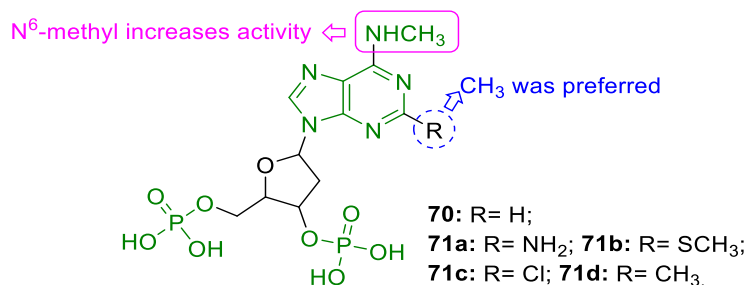


Fig.49. Chemical structures of **70** and **71**.

Based on the SAR profile of a series of non-cyclic fatty chain nucleotide analogues explored as P2Y₁ receptor antagonists, Kim et al. [84] found that compound **72e**, a full antagonist for P2Y₁ receptor, was the most potent one with IC₅₀ value of 0.48 μM, while its corresponding 2-H derivative, compound **72d** had a weaker effect than **72e** (IC₅₀: 1.09 ± 0.51 μM). Compound **72a** and its 2-chlorine analogue compound **72b** with longer alkyl chain compared to **72e**, were moderately potent antagonists with the IC₅₀ values of 1.60 μM and 0.84 μM, respectively (Fig. 50).

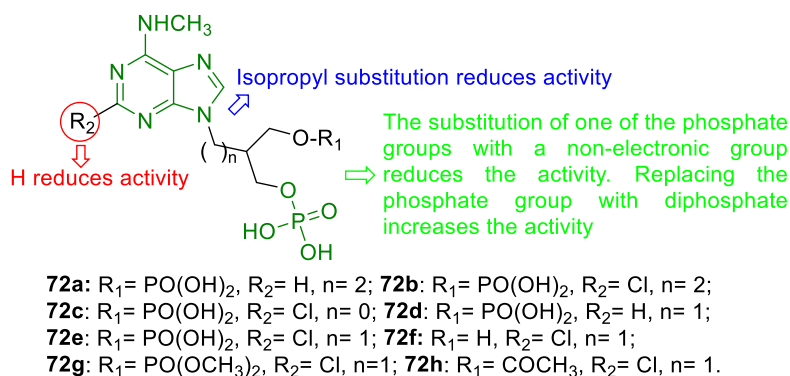


Fig.50. Chemical structures of P2Y₁ receptor antagonists **72**.

Lourenco et al. [85] synthesized a series of *N, N'* - disubstituted thioureas derivatives and analyzed their antithrombotic property (Fig. 51). The most active thioureas compounds **73a**, **73b**, **73c** and **73d** (IC₅₀: 29.00-84.00 μM) compared to aspirin (IC₅₀: 30.56 ± 0.40 μM) directly affected the formations of PGE₂ and TXA₂, reduced thromboxane B₂ (TXB₂) production and directly inhibited COX-1 to achieve antiplatelet effect in human thrombus model.

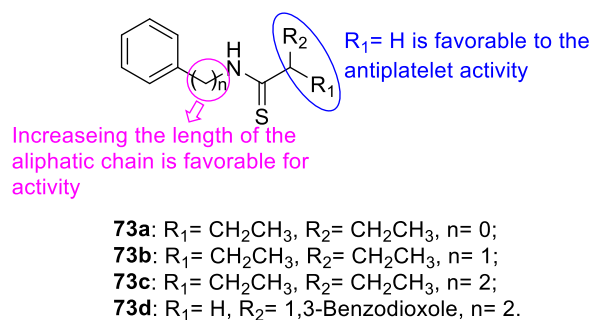


Fig.51. Chemical structures of *N, N*-disubstituted thioureas derivatives **73**.

In order to search for new platelet aggregation inhibitors, Xie et al. [86] designed and synthesized two series of 1,4-benzodioxazine derivatives based on the previous antiplatelet drugs **LX2421**, which had effective antiplatelet activity and no risk of bleeding [87,88]. By introducing 1,4-benzodioxazine to replace the indole structure in **LX2421**, new 1,4-benzodioxazine derivatives were established as the platelet aggregation inhibitors (Fig. 52). Compounds **74** significantly inhibited platelet aggregation induced by ADP and thrombin, with IC_{50} values of 41.70 μM and 22.20 μM , respectively, stronger than **LX2421**. Moreover, it had a strong antagonistic activity of GPII/IIIa with an IC_{50} of 2.30 μM , better than GPIIb/IIIa antagonist RGD.

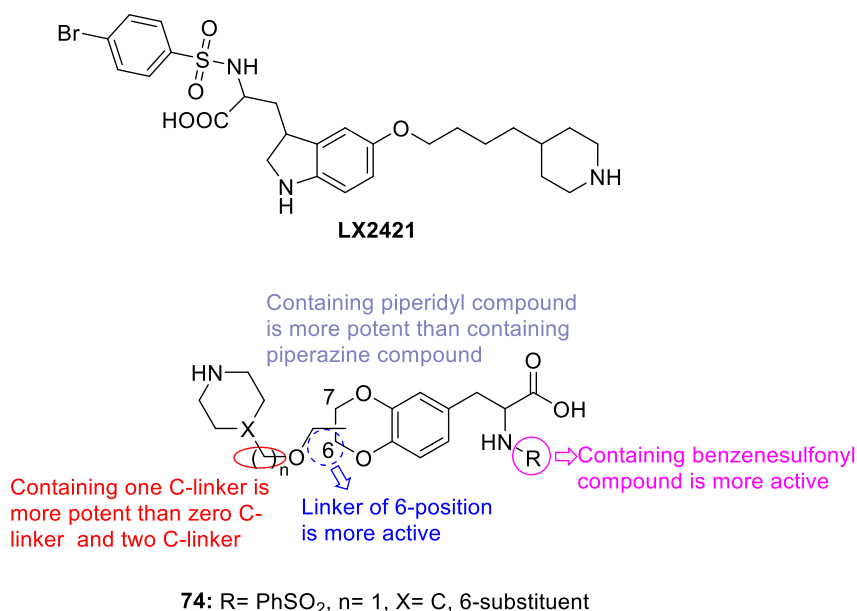


Fig.52. Chemical structures of 1,4-benzodioxazine derivatives **74**.

Zhang et al. [89] designed and synthesized a range of 2-hydroxytetrahydrothienopyridine derivatives using the metabolite of prasugrel as the lead compound (Fig. 53). In the pharmacological experiment of antiplatelet aggregation after oral administration of these compounds in rats, most of the

compounds except compound **75e** showed better anti ADP-induced platelet aggregation activity compared with prasugrel. Compounds **75a-75d** and **75f** showed similar antiplatelet aggregation activity as prasugrel (platelet aggregation (PA): $22.83 \pm 4.99\%$), and compound **75b** (PA: $18.50 \pm 7.94\%$) showed slightly stronger activity than prasugrel.

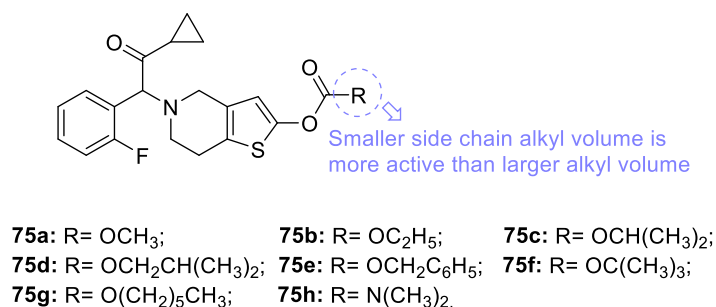


Fig.53. Chemical structures of 2-hydroxytetrahydrothienopyridine derivatives **75**.

Jaiswal et al. [90] synthesized a bunch of 2-oxo-2-phenylethylidene linked 2-oxo-benzo [1,4] oxazine analogues **76** (Fig. 54). Compared with the standard drug aspirin (IC₅₀: $21.34 \pm 1.09 \mu\text{g/mL}$), these compounds exhibited moderate to good inhibition activity on platelet aggregation induced by arachidonic acid. **76a-76f** were considered promising platelet aggregation inhibitors, in which **76d** (IC₅₀: $17.96 \pm 0.18 \mu\text{g/mL}$) and **76f** (IC₅₀: $16.96 \pm 0.18 \mu\text{g/mL}$) displayed the best activity. Based on docking analysis, **76d** and **76f** had a strong binding affinity with anti-platelet target COX-1.

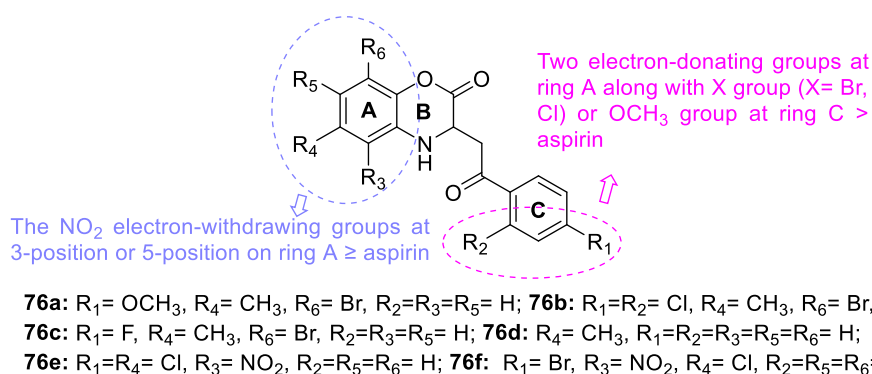


Fig.54. Chemical structures of 2-oxo-2-phenylethylidene linked 2-oxo-benzo [1,4] oxazine analogues **76**.

Using nicotinic acid as the precursor, He et al. [91] designed and synthesized 10 nicotinic acid derivatives by coupling different junction bridges with acetylsalicylic acid and nicotinic acid. In vitro biological activity study showed that all compounds

had certain anti-platelet aggregation activities. The inhibitory activity of compounds **77a-77e** on platelet aggregation is higher than that of niacin (aggregation inhibition rate (AIR): 1.82%), **77a** (AIR: 41.13%) and **77b** (AIR: 40.73%) were stronger than that of the control drug aspirin (AIR: 32.26%), while compounds **78a-78e** are lower than that of aspirin (Fig. 55). These compounds inhibited TXA₂ synthesis and promoted PGI₂ synthesis or blocked COX-1 activity, thereby playing an anti-platelet aggregation role.

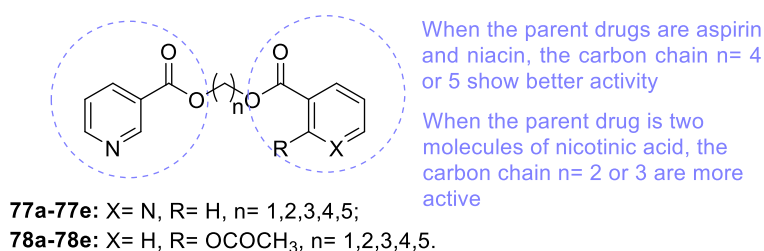


Fig.55. Chemical structures of nicotinic acid derivatives **77** and **78**.

Zhou et al. [92,93] synthesized a series of NO donor aspirin derivatives (**79a-79n**, **80a-80h**) by connecting aspirin with furoxans nitrogen oxide and nitrate via ferulic acid or hydroxycinnamic acid (Fig. 56). In the platelet aggregation test, **79a-79c** (AIR: 12.10%, 14.80% and 19.70%), **79i** (AIR: 15.70%) and **79l-79n** (AIR: 8.00%, 16.00% and 28.70%) reduced the platelet aggregation comparable to aspirin (AIR: 14.70%). In the rat model of arteriovenous bypass thrombosis, **79i** (AIR: 16.00%) inhibited arteriovenous bypass thrombosis in rats comparable to aspirin (AIR: 19.20%). The complexes **80b**, **80d**, **80e**, **80f**, **80g** and **80h** decreased platelet aggregation significantly compared with the negative control group ($p < 0.05$). The activity of **80f** was significantly higher than that of aspirin ($p < 0.01$). **80f** inhibited arteriovenous bypass thrombosis in rats with a better inhibition rate (43.90%) than aspirin (28.05%), and significantly increased TXB₂ and 6-Keto-PGF_{1 α} levels of antithrombotic activity.

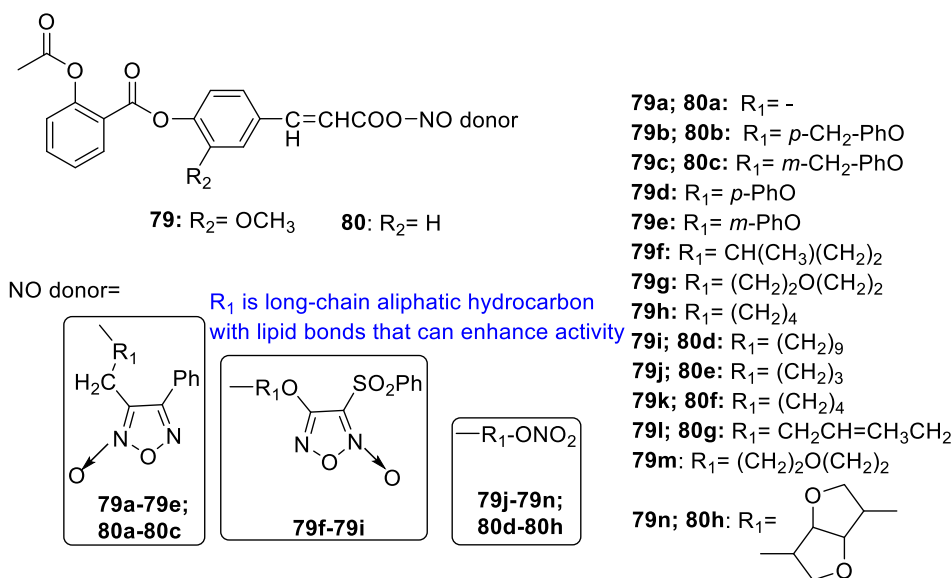


Fig.56. Chemical structures of hydroxycinnamic acid-combined aspirin derivatives **79** and **80**.

Picotamide, a potent platelet inhibitor, can simultaneously inhibit TXA₂ synthase and PGH₂ receptor of platelet [94]. Wei et al. [95] synthesized a series of picotamide analogs (**81a-81j**) and assessed their anti-platelet aggregation activity in vitro (picotamide and aspirin as positive controls) by Born Turbidimetry (Fig. 57). Most of them (**81a**, **81b**, **81d**, **81f**, **81i**, **81j**) had effective inhibitory ability, in which compound **81b** was the most potent one (IC₅₀: 0.35 μM), superior to picotamide (IC₅₀: 0.76 μM) and aspirin (IC₅₀: 0.53 μM).

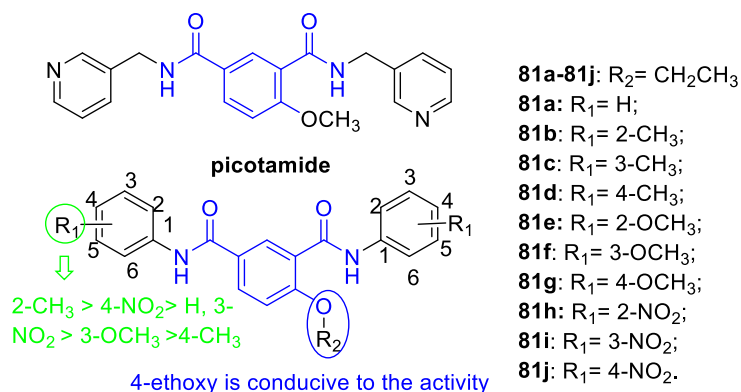
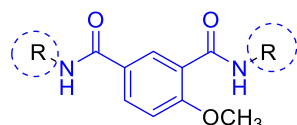


Fig.57. Chemical structures of picotamide analogs **81**.

Liu et al. [96] synthesized 4 series of picotamide derivatives (Fig. 58): series 1 (ortho-substituted phenyl: **82a-82j**), series 2 (inter-substituted phenyl: **83a-83k**), series 3 (para-substituted phenyl: **84a-84l**), and series 4 (aromatic without substituted group and aromatic heterocyclic substituted groups). The anti-platelet aggregation activities in vitro were evaluated by Born method and the results showed that

compounds **82c**, **82d**, **82i**, **82j**, **83g**, **84a**, **84c**, **84d**, **84f**, **84h**, **84i**, **85b** and **85c** exhibited better anti-platelet aggregation activity than the control drug picotamide (IC_{50} : 0.76 μ M). The compound **82i** (IC_{50} : 0.02 μ M), compound **83g** (IC_{50} : 0.49 μ M), compound **84i** (IC_{50} : 0.49 μ M) and compound **85c** (IC_{50} : 0.24 μ M) had the strongest activity in their respective series.

Series 1: $CH_2Ph > I > CH_2CN > Br > F > \text{the others}$



Series 2: no obvious anti-platelet aggregation activity, strong electron-withdrawing substituents, halogens and weak electron-donating substituents in meta-position are not conducive to activity

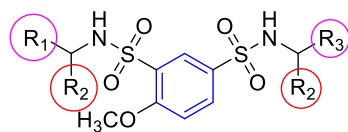
Series 3: $O(CH_2)_3CH_3 > Br > F > CH_3 > COCH_3 > NO_2 > \text{the others}$ Series 4: $(CH_2)_2Ph > CH_2Ph > Ph > \text{the others}$

- 82**: R= *ortho*-Ph,
82a-82j (F, Cl, Br, I, NO₂, CH₃, OCH₃, COOH, CH₂Ph, CH₂CN);
83: R= *meta*-Ph,
83a-83i (F, Cl, Br, I, NO₂, CH₃, OCH₃, COCH₃, OCH₂CH₃),
83j: R= CH₂-Ph-*meta*-Cl, **83k**: R= CH₂-Ph-*meta*-Br;
84: R= *para*-Ph,
84a-84i (F, Cl, Br, I, NO₂, CH₃, OCH₃, COCH₃, OCH₂CH₃, O(CH₂)₂CH₃, CH₂CH(CH₃)₂, O(CH₂)₃CH₃);
85a: R= Ph; **85b**: R= CH₂-Ph; **85c**: R= (CH₂)₂-Ph; **85d**: R= CH₃CH-Ph;
85e: R= Ph-Ph; **85f**: R= pyridine; **85g**: R= CH₂-oxole; **85h**: R= (CH₂)₂-2,3-Benzofuran.

Fig.58. Chemical structures of compounds **82-85**.

A series of 4-methoxy-1,3-benzenedisulfonamide compounds (**86a-86j**) were designed [97] based on the SAR of platelet inhibitor picotamide derivatives (Fig. 59). According to Born turbidimetry, an anti-platelet aggregation activity study was conducted and showed that all compounds except **86g** and **86j** had various degrees of inhibitory activities against ADP-induced rabbit platelet aggregation in vitro, with aspirin, picotamide and clopidogrel as positive controls. Compound **86b** was the most effective inhibitor at 1.30 μ M, with IC_{50} value of 0.46 μ M, and its inhibition rate of 62.30% was much higher than that of aspirin (16.47%) and picotamide (26.01%). The compounds (**86h** and **86i**) substituted with methyl groups to methylene had no obvious activity in either R or S configurations.

R₁, R₃ activity: benzene ring > furan ring;
Substituent for R₁: F > Br > OCH₃



The methyl of R configuration or S configuration both have little active

- 86a:** R₁= Ph, R₂= H, R₃= Ph; **86f:** R₁= 4-Cl-Ph, R₂= H, R₃= 4-Cl-Ph;
86b: R₁= 2-F-Ph, R₂= H, R₃= 2-F-Ph; **86g:** R₁= 4-OCH₃-Ph, R₂= H, R₃= 4-OCH₃-Ph;
86c: R₁= 2-Cl-Ph, R₂= H, R₃= 2-Cl-Ph; **86h:** R₁= Ph, R₂= CH₃(R), R₃= Ph;
86d: R₁= 3-Br-Ph, R₂= H, R₃= 3-Br-Ph; **86i:** R₁= Ph, R₂= CH₃(S), R₃= Ph;
86e: R₁= 4-F-Ph, R₂= H, R₃= 4-F-Ph6 **86j:** R₁= 2-furyl, R₂= H, R₃= 3-furyl.

Fig.59. Chemical structures of 4-methoxy-1, 3-benzenedisulfonamide compounds **86**.

He et al. [98] synthesized a cyclic precursor drug **87a-87d** based on RGD (Arg-Gly-Asp) peptidomimetics **88a-88d** with different ring sizes ($[\text{CH}_2]_n = 1, 3, 5$ and 7) and evaluated the antithrombotic activity of prodrugs **87a-87d** and the parent compounds **88a-88d** by platelet aggregation test, with RGDF peptide (IC_{50} : 12.00 μM) as a positive control [99] (Fig. 60). The results showed that the antithrombotic activity of **88a-88d** increased with the increase of methylene number, and the antithrombotic activity of prodrug **87a-87d** was lower than that of the parent compound **88a-88d**, indicating that the cyclic prodrug **87a-87d** had to be converted into the parent drug by esterase to exert antithrombotic effect. Cyclic prodrug **87d** (IC_{50} : $0.40 \pm 0.03 \mu\text{M}$) was the best antithrombotic one, which is the premise for its transformation into the parent drug **88d** (IC_{50} : $0.20 \pm 0.01 \mu\text{M}$).

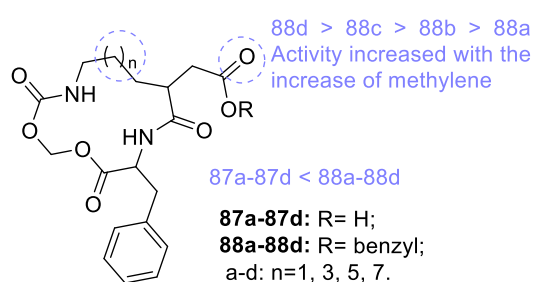


Fig.60. Chemical structures of cyclic precursor drug **87** and **88**.

The new aspirin derivatives formed by the connection of NO donor (inhibition of platelet aggregation) with the antiplatelet drug aspirin have strong antithrombotic activity and low gastrointestinal side effects. Therefore, Zhou's group [100] coupled 3-aryl-1, 2, 3, 4-oxtriazole-5-imine (with antiplatelet activity) as a NO donor to aspirin to synthesize a battery of new NO donor-type aspirin derivatives (**89a-89I**) and

found that **89f**, **89k**, **89l** could not merely inhibit platelet aggregation induced by ADP in vitro (with aspirin as positive control and 0.10% DMSO as negative control) but also effectively inhibit the formation of pulmonary thrombosis in vivo (with CMC-Na as negative control and aspirin as positive control), and **89f** had the best inhibition activity in vitro with inhibitory rate of 30.70%. In summary, compounds **89f**, **89k** and **89l** had effective antithrombotic activity whether in vivo or in vitro, and further investigation is needed on their substituents and positions (Fig. 61).

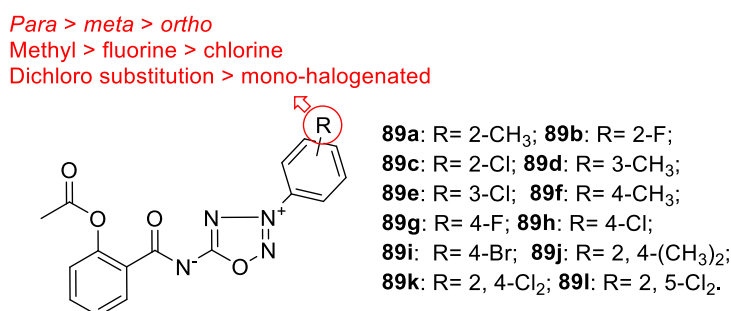


Fig.61. Chemical structures of NO donor-type aspirin derivatives **89**.

Ferulic acid is an effective ingredient in traditional Chinese medicine to promote blood circulation and remove blood stasis. Modern pharmacology has shown that ferulic acid can effectively inhibit the release of serotonin in platelets to display its antithrombotic activity [101]. Li et al. [102] synthesized six ferulic acid derivatives (**90a-90f**), using ozagrel and dazoxiben (with selective inhibition for TXA₂ synthase) as model compounds and ferulic acid as lead compounds. All compounds showed significant inhibitory activities on the ADP-induced platelet aggregation and possessed better activity than ozagrel in vivo. Among them, compounds **90a** and **90e** were the best, with their respective inhibition rates of 83.10% and 91.40%, which were 3.40 and 3.70 times of ozagrel. Moreover, application of the Energy minimizer (MOE software) to calculate the distance between the basic nitrogen atom and the carboxyl group of the ferulic acid derivatives at the lowest energy indicated that the distance of both **90a** and **90e** were less than 1.0 nm, similarly to ozagrel and dazoxiben.

Compound **91** which was synthesized by the structural characteristics of ferulic acid and TXA₂ synthase inhibitor had a pronounced inhibitory effect on ADP-induced

platelet aggregation. Inspired by pyrazine introduced as a pharmacophore with good antiplatelet aggregation activity in previous literature [103], Ma et al. [104] synthesized a series of new [(pyrazine-3-yl) methoxy] aromatic acid derivatives (**92a-92f**) by replacement of pyridine with pyrazine and different substituted methyl aromatic acid esters in compound **91**. The in vitro antiplatelet aggregation activity of **92a-92f** was evaluated by Born turbidimetric assay, with ozygrel and ferulic acid as positive control, the data displayed that all compounds had effective suppressions on ADP-induced platelet aggregation in rabbit model. Compounds **92a** (IC₅₀: 1.14 mM) and **92e** (IC₅₀: 1.81 mM) had better inhibitory activity than ozagrel (IC₅₀: 2.85 mM) and ferulic acid (IC₅₀: 19.58 mM), and could be comparable to the compound **91** (IC₅₀: 1.76 mM) (Fig. 62).

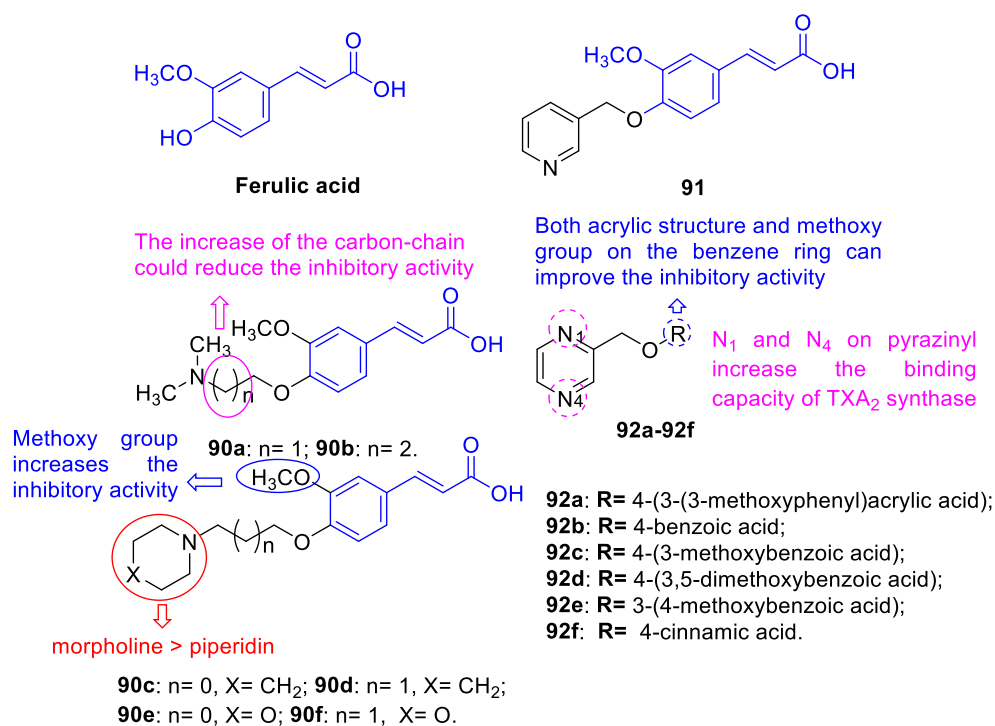


Fig.62. Chemical structures of ferulic acid derivatives **90-92**.

Exposure of GPIIb/IIIa receptors on the surface of platelets can promote the binding of fibrinogen molecules to platelets, causing the aggregate of platelets and leading to thrombosis. Liu et al. [105] synthesized a series of compounds with inhibitory activity of GPIIb/IIIa receptor by different chemical substitutions at 7-position and 4-position of 1,4-benzoxazine-3(4H)-one (Fig. 63). In the anti-platelet aggregation activity test of rabbit arterial blood samples, using aspirin and ticlopidine

as positive controls, the activities of **93c** and **93d** (IC_{50} : 8.99 μ M and 8.94 μ M, respectively) with a shorter branched chain or ring structure on C-7 were the best, due to smaller steric hindrance. Molecular docking experiments demonstrated that **93c** and **93d** could interact with the GPIIb/IIIa receptor and indicated that 1, 4-benzoxazine-3(4H)-one was a promising inhibitor for platelet aggregation.

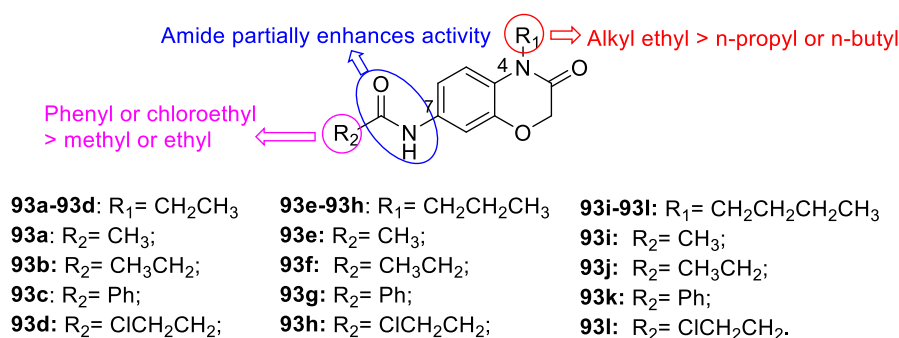


Fig.63. Chemical structures of 1,4-benzoxazine-3(4H)-one substitutions **93**.

The dihydropyridazinone compound **CCI-17810** has an effective inhibitory effect on platelet aggregation caused by factors such as ADP and platelet activating factor (PAF). In order to develop less toxic and more potent inhibitors, two clusters of dihydropyridazinone derivatives, **94a-94g** and **95a-95e**, were synthesized by Zhao et al. [106] based on **CCI-17810**. Born turbidimetry method was used to determine the activity of these derivatives by an anti-platelet aggregation test (with **CCI-17810** as positive control) in vitro, which showed that **95a-95e** and other two compounds **94f**, **94g** had inhibitory effects on ADP-induced platelet aggregation in healthy people. Compound **95b** exerted the strongest inhibitory activity, with an IC_{50} value of 0.008 μ M, more than 60 times than that of the positive control **CCI-17810** (IC_{50} : 0.49 μ M). Further analysis of the SAR showed that the anti-platelet aggregation activity of **95** was generally stronger than that of **94**.

Taking **CCI-17810** as the lead compound, Wu et al. [107] synthesized 18 6-(substituted phenyl)-4,5-dihydro-3(2H)-pyridazinone compounds. Preliminary in vitro pharmacological test results showed that most of the target compounds except **96m** and **96q** had varying degrees of inhibition for ADP-induced platelet aggregation in New Zealand rabbits, stronger than the lead compound **CCI-17810**. Compound **96e** was the most effective with IC_{50} value of 2.50 μ M, much better than that of the

positive control **CCI-17810** (IC_{50} : 12.50 μ M) (Fig. 64).

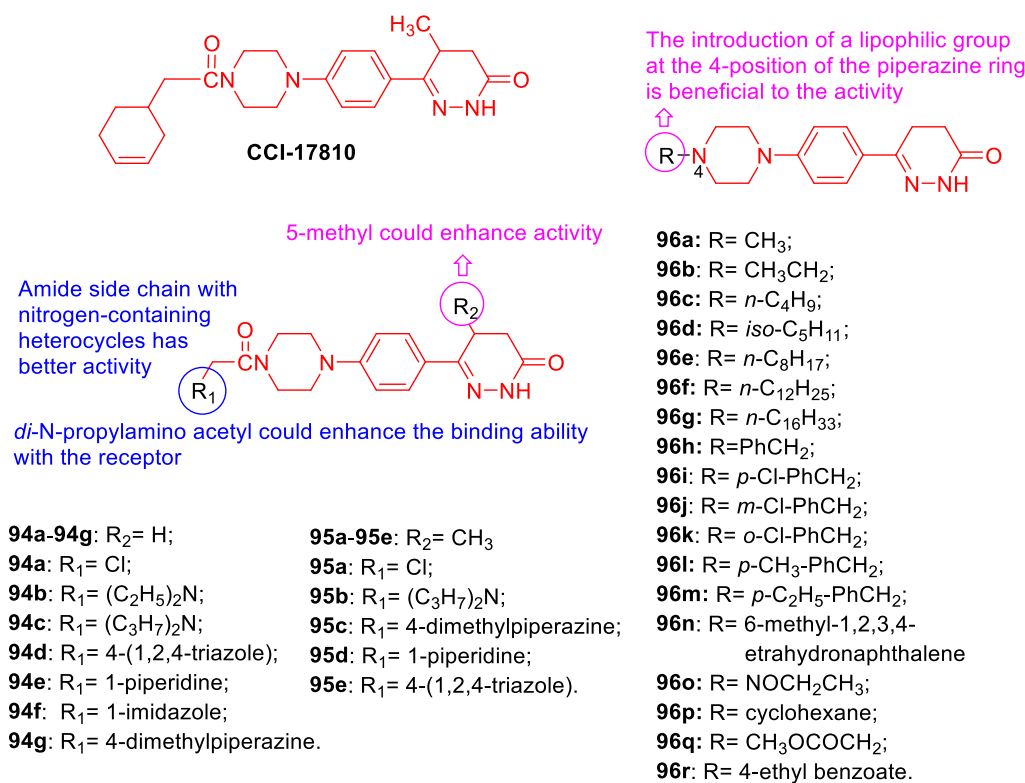


Fig.64. Chemical structures of dihydropyridazinone derivatives **94-96**.

In addition, because pyridazinones were effective inhibitors for platelet aggregation, and the pyridazinone derivatives 6- [4- (4'-pyridylaminobenzene)]-4,5-dihydro-3 (2H) pyridazinones (**MCI-154**) had strong cardiotoxic and antiplatelet aggregation activity, two types of compounds with similar structure to **MCI-154** were designed and synthesized [108], namely 6-(4-substituted chloroacetamido) phenyl-4,5-dihydro-3(2H)-pyridazinone and 6-(4-substituted chloroacetamido) phenyl-5-methyl-4,5-dihydro-3(2H)-pyridazinone, in order to explore the underlying SAR of pyridazinones and find more potent and selective inhibitors (Fig. 65). Compounds **97a-97h** (with **MCI-154** as a control) were conducted by an in vitro pharmacological experiment, and most compounds had different degrees of activity against human platelet aggregation in vitro, compound **97a** had the strongest activity (IC_{50} : 0.03 μ M) with more 10 times than that of **MCI-154** (IC_{50} : 0.36 μ M). The activity of compounds **97f-97h** (IC_{50} : 0.085, 0.050, 0.060 μ M, respectively) was also superior to **MCI-154**, and **97b-97e** were comparable to **MCI-154**.

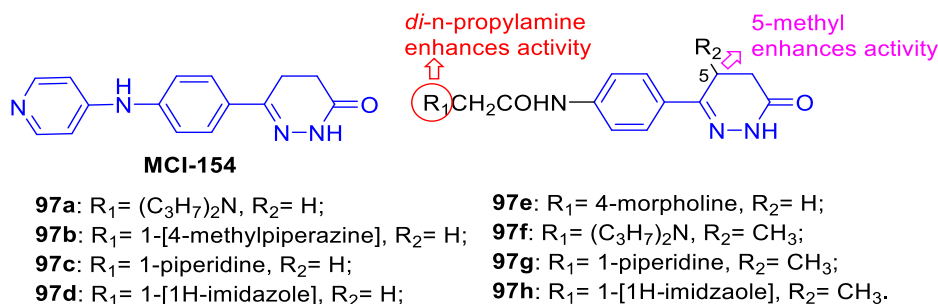


Fig.65. Chemical structures of MCI-154 and analogues **97**.

Based on previous research [109], Nikitina et al. [110] synthesized enantiomerically pure sulfoxide **98** to study its cytotoxicity and ability to inhibit bleeding in vitro (Fig. 66). In addition, the possibility of **98** as a platelet concentration stabilizer was investigated, the results showed that **98** could almost completely inhibit arachidonic acid, collagen, and epinephrine-induced platelet aggregation. Finally, it was confirmed that the anticoagulant property of **98** was related to its ability to stabilize cell membrane by interaction of outer phospholipids and van der Waals forces. In conclusion, compound **98** is expected to be a promising drug for stabilizing platelet blood products, treating and preventing thrombosis.

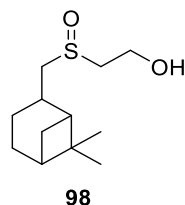


Fig.66. Chemical structures of enantiomerically pure sulfoxide **98**.

Giorgio et al. [111] designed and synthesized a series of new compounds **99** and its analogues **100**, which were tested in vitro using acetylsalicylic acid (ASA), cilostazol, and milrinone as reference compounds against ADP, collagen, and Ca²⁺ ionophore A23187 in platelet-rich plasma (PRP) inhibitory activity of human platelet aggregation (Fig. 67). The new compounds **99a-99g** were proved to be inhibitors of human platelet aggregation in vitro, significantly more active (IC₅₀ on ADP: 0.90 ± 0.36 μM-6.10 ± 2.10 μM) than the reference compounds, because they increased the platelet cAMP level by specifically inhibiting phosphodiesterase 3 (PDE3) to display antiplatelet activity, and they were much more active than their analogues **100** (IC₅₀

on ADP: $74.80 \pm 10.00 \mu\text{M}$ and $95.00 \pm 15.00 \mu\text{M}$, respectively).

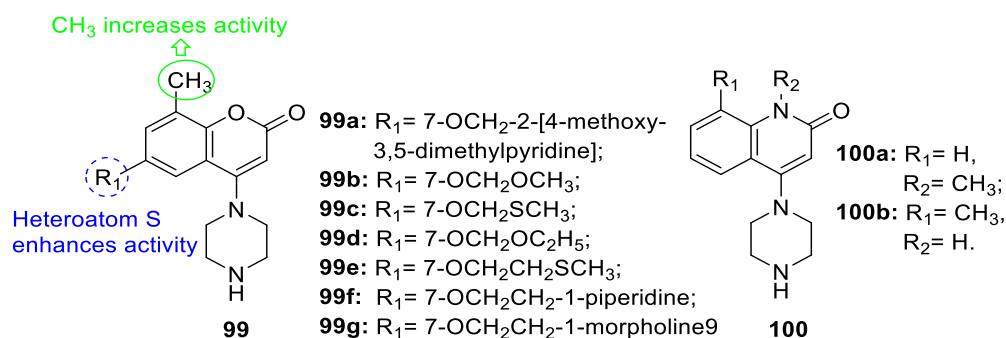


Fig.67. Chemical structures of inhibitors **99** and **100**.

In view of the existing drugs such as tirofiban, eptifibatid and abciximab with the disadvantage of thrombocytopenia, Pavel et al. [112] developed and designed two types of $\alpha_{\text{IIb}}\beta_3$ antagonists which can integrate into closed or open forms of the receptor pocket (Fig. 68). Various theoretical methods such as QSAR, docking studies and pharmacophore models based on structure and ligands have been applied to screen out and provide 4 compounds (**101a-101d**) and 3 compounds (**102a-102c**) as ligands for the open and the closed form of the target protein, respectively. The in-vitro assay of inhibition on ADP-induced platelet aggregation indicated that **101c** (IC_{50} : 6.20 nM) and **102b** (IC_{50} : 11.00 nM) were the most potent in their respective series, as well as more effective than the antithrombotic tirofiban (IC_{50} : 32.00 nM) commonly used in clinical practice.

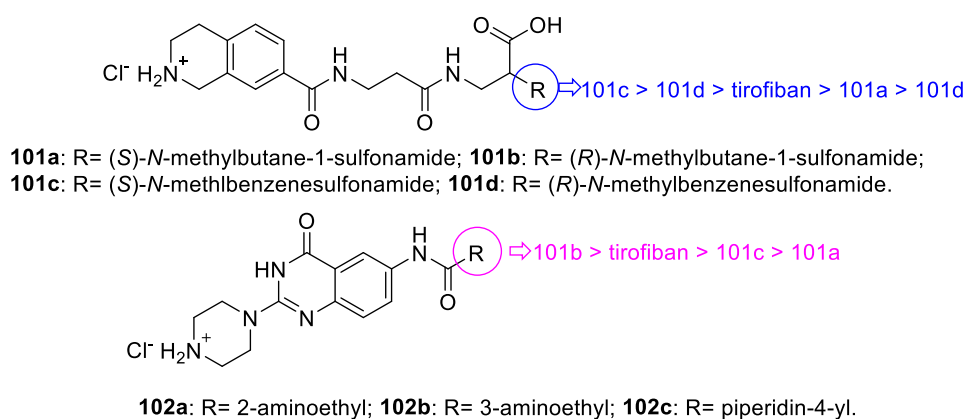


Fig.68. Chemical structures of $\alpha_{\text{IIb}}\beta_3$ antagonists **101** and **102**.

Compared with other antiplatelet drugs, clopidogrel has the advantages of good efficacy, low cost, and small adverse reactions, but however it also has defects, such as slow onset, large individual variation in platelet inhibition, thrombotic

thrombocytopenic purpura and hemolytic uremic syndrome. To overcome the above weakness, Chen et al. [113] designed and synthesized clopidogrel analogues **103** and **104**, and used clopidogrel as a control for determination of anti-platelet aggregation activity (Fig. 69). Preliminary bioactivity test results showed that **103** and **104** had a certain degree of anti-platelet aggregation effects, among them, **103b** and **103g** were particularly pronounced and their inhibition percentage of platelet aggregation in rats were 46.10% and 38.20%, respectively. Although the activity of these compounds was less active than the reference clopidogrel on anti-thrombosis, it provided important information for the next step of research. What is noteworthy is that inhibition of platelet aggregation is not “the stronger the better”, since excessive inhibition would increase the risk of bleeding.

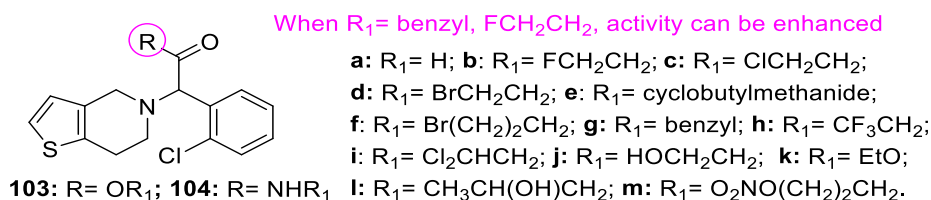


Fig.69. Chemical structures of clopidogrel analogs **103** and **104**.

Enlightened by various potency of quinoline, benzimidazole and oxadiazoles derivatives in pharmacology, Bharadwaj et al. [114] designed to combine these three drug scaffolds and synthesized benzimidazole-containing quinolinyl oxadiazoles, as well as explored their anticoagulant and antiplatelet activities by conducting a series of experiments and detecting antiplatelet and toxicity properties in RBC cells (Fig. 70). Among them, compound **105a** and **105b** were nontoxic and exhibited anticoagulant potency by enhancement in clotting time, further, **105a** also inhibited 93.4% of platelet aggregation induced by epinephrine at the concentration of 30 µg.

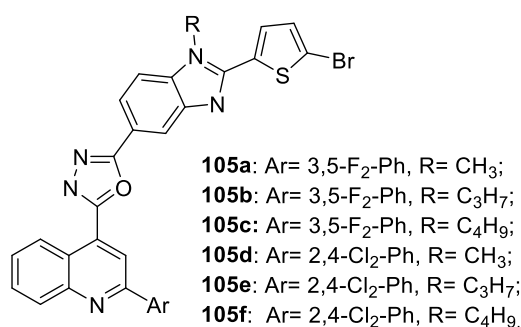


Fig.70. Chemical structures of benzimidazole-containing quinolinyl oxadiazoles compound **105**.

Polyethylene glycol (PEG) is not widely used for covalent modification of anti-thrombosis because of its potential steric complications, whereas dendritic scaffolds confer powerful spatial control to overcome the disadvantage of PEG. Given this, Kim et al. [115] used the third generation (G3) PAMAM dendrimer to conjugate nucleoside moieties and PEG chains, in order to study the effect of PEG chains on the anti-thrombosis bioactivity (Fig. 71). Cyclic AMP accumulation assay showed that the dendrimer conjugate **106a** with 11 PEG₇₅₀ chains was 5-fold more active than **106b** with 4 PEG₂₀₀₀ chains in the A_{2A} AR-mediated cyclic AMP formation, while ADP-induced platelet aggregation experiment also displayed that the inhibition of **106a** (IC₅₀: 0.15 ± 0.053 μM) on platelet aggregation was stronger than that of macromolecule **106b** (IC₅₀: 0.49 ± 0.14 μM), suggesting that longer PEG chains were not favorable for the potency due to steric hindrance.

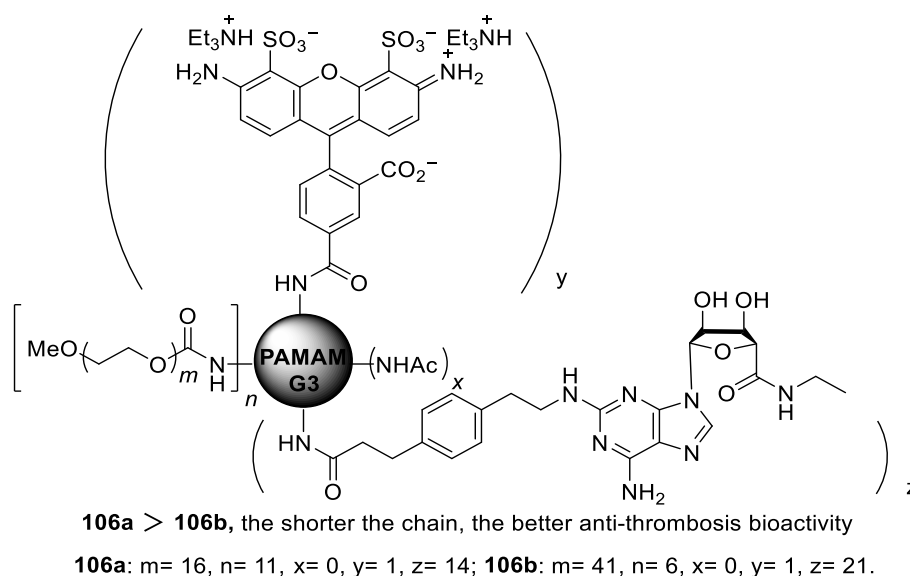


Fig.71. Chemical structures of dendrimer conjugates **106**.

Based on the platelet inhibitors reported in the literature described above, we summarized their respective targets and structural characteristics, and found that the main targets were GPIIb/IIIa, TXA₂ and P2Y₁, as shown in the Table 2.

Table 2. Platelet inhibitor compounds and their targets

NO.	Compound name	Target	Structural characteristics
1	59	$\alpha_{IIb}\beta_3$	benzo-oxazin-one
2	60	GPIIb/IIIa	benzo-oxazin-one
3	66	PAE	lebetin
4	67~71	P2Y ₁	adenosine 3',5'-diphosphate
5	72	P2Y ₁	fatty chain nucleotide analogues
6	73	COX-1	N, N' - disubstituted thioureas
7	74	GPIIb/IIIa	benzodioxazine
8	75	P2Y ₁	2-hydroxytetrahydrothienopyridine
9	76~78	COX-1	phenylethylidene
10	79~80	TXA ₂	hydroxycinnamic acid combine aspirin
11	82~86	TXA ₂	picotamide
12	87~88	GPIIb/IIIa	cyclic precursor
13	90~92	TXA ₂	ferulic acid
14	93	GPIIb/IIIa	benzoxazine-one
15	99~100	PDE3A	/
16	101~102	$\alpha_{IIb}\beta_3$	/
17	106	GPCRs	Polyethylene glycol

3.3 Fibrinolytics

Using diosgenin as the lead compound, Fu et al. [116] synthesized 9 compounds and examined their antithrombotic activities by using thrombogenesis model of arteriovenous bypass in rats (Fig. 72). Compounds **107h** (quality of thrombosis = 8.90 ± 4.10 mg) and **107i** (quality of thrombosis = 7.60 ± 3.20 mg) with salicylic group showed good activities compared to aspirin (quality of thrombosis = 10.50 ± 7.10 mg) in anti-thrombotic activity screening test. Moreover, the C-3 position in the parent structure of diosgenin was modified to obtain a series of derivatives **108** [117], and evaluated their anti-thrombotic activities. 3β - diosgeninyl monosuccinate (quality of

thrombosis = 18.70 ± 9.30 mg) had the strongest inhibitory effect, similar to that of aspirin (quality of thrombosis = 15.40 ± 8.10 mg), indicating that the modification of appropriate hydrophilic groups on the C-3 hydroxyl group could improve the antithrombotic activity of diosgenin.

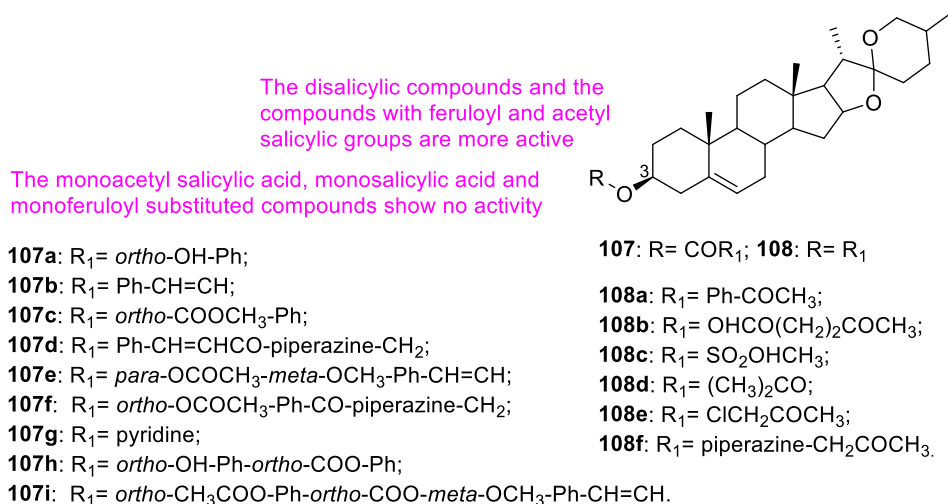


Fig.72. Chemical structures of diosgenin derivatives **107** and **108**.

Chondroitin sulfate (CS), a sulfated polysaccharide, is a class of highly complex biomolecules called glycosaminoglycans, which can be covalently attached to the nuclei of proteins to form proteoglycans. The size and quantity of CS chains, binding site and degree of sulfation make CS a bunch of different molecules that exert various activities. Based on the enormous complexity of the molecular structure and functions of CS in different species, Cavalcante et al. [118] extracted sulfated chondroitin sulfate (sCS: **109a-109c**) from the head of the Pacific white shrimp (Fig. 73). In the anticoagulant assay of sCS, the APTT and PT were determined, with pig-heparin (standard anticoagulant) as control, the result showed that sCS could not affect APTT and PT. In the antithrombotic experiment evaluated by rat venous thrombotic model, the control group only accepted saline solution and thrombotic formation rate reached 100%, conversely, administration with sCS and heparin led to a dramatic decrease in the thrombus weight and the inhibition rate of both agents was about 70%.

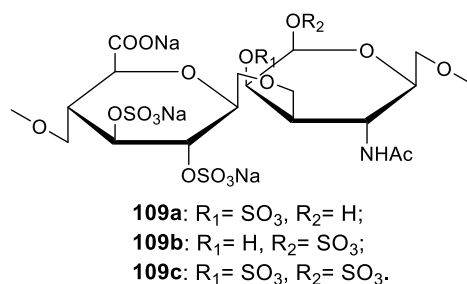


Fig.73. Proposed structures for the peculiar disaccharide unit found in **sCS**.

Given involvement of plasminogen activator inhibitor (PAI)-1 in thrombogenesis, Izuhara et al. [119] synthesized a novel PAI-1 inhibitor **110**, by the extensive research on the SAR of the lead compound **TM5007** and assessed its effectiveness on blood clots by rat model and cynomolgus monkey (a representative of nonhuman primates), aiming to reduce differences between animal trials and practically clinical studies (Fig. 74). In rat model, the clot weights after administration of 10 and 50 mg/kg of **110** (60.90 ± 3.00 and 56.80 ± 2.80 mg) were lower than that in no-treated rats (72.50 ± 2.00 mg); while in the monkey thrombosis model, the occlusion time of **110** and clopidogrel group (53.90 ± 19.90 mins and 39.40 ± 25.80 mins, respectively) pronouncedly decreased, compared with no-treated group (119.00 ± 17.40 mins).

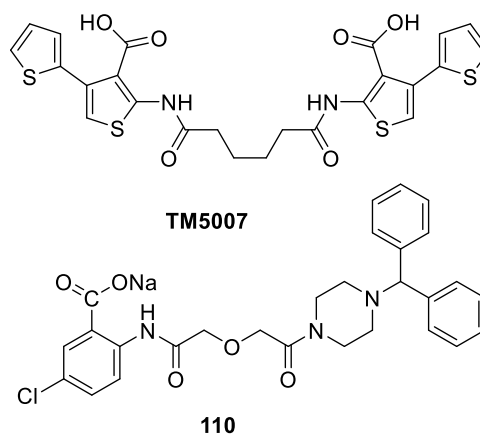


Fig.74. Chemical structures of PAI-1 inhibitor **TM5007** and **110**.

Inflammation can promote the formation of thrombosis, and P-selectin can cause white blood cells and platelets to adhere to the inflammation or injury site, therefore, inhibiting P-selectin and inflammation can effectively treat thrombosis-related diseases. Screening 126 derivatives (with antithrombotic and anti-inflammatory activities) of β -carboline 3-carboxylic acid, tetrahydro- β -carboline-3-carboxylic acid and indoloquinolizine to dock with the active pocket of P-selectin by rational

computer assistant, Wu et al. [120] constructed the optimized compound (2-(3-(hydroxymethyl)-9H-pyrido[3,4-b] indol-1-yl) ethyl)-L-phenylalanine (**111**) by comparison of the binding energy and analyzing the pharmacophore of these derivatives (Fig. 75). Compound **111** could directly bind to P-selectin and showed dose-dependent behavior, and the thrombus weight of the rats and mice accepted 200 nmol/kg of **111** is equivalent to that of 167 $\mu\text{mol/kg}$ and 240 $\mu\text{mol/kg}$ of aspirin, respectively, suggesting that **111** was favorable for discovering novel, potent antithrombotic leads of P-selectin inhibitors.

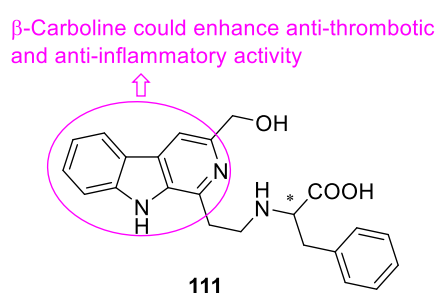


Fig.75. Chemical structures of (2-(3-(hydroxymethyl)-9H-pyrido[3,4-b] indol-1-yl) ethyl)-L-phenylalanine (**111**).

Although recombinant *t*-PA, SK and UK are widely used as thrombolytic agents to inhibit clot formation in clinical practice, the safety of these drugs is low and may bring undesirable results such as severe bleeding complications, therefore, it is necessary to develop a new type of fibrinolytic with high safety. Silver and gold nanoparticles can be introduced into antithrombotic agents to be developed as anticoagulants. Harish et al. [121] isolated xylan from wheat bran (WB) by alkaline treatment [122] which was used to prepare silver nanoparticles with fibrinolytic activity, WB-xylan AgNPs. DPPH free radical scavenging experiment and fibrin plate assay proved that WB-xylan AgNPs not only had favorable free radical scavenging activity but also displayed high fibrinolytic activity (Fig. 76). In addition, in order to further explore the potential of WB-xylan AgNPs to treat thrombosis, blood clot was on a glass plate in vitro (as a negative control), uncoagulated blood was gained by addition of sodium citrate (as a positive control), then WB-xylan AgNPs were added to the blood clot to result in dissolution within 5 minutes, in line with the fibrin plate assay and positive control. However, its specific role in fibrinolysis remains unclear and the safety is needed to further study.

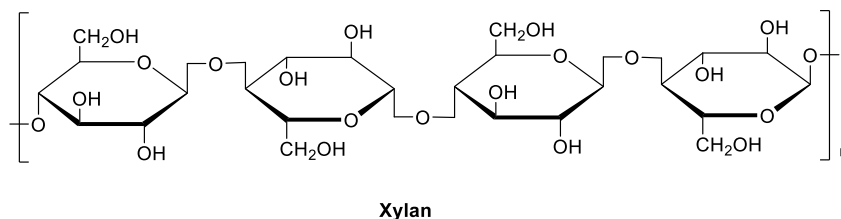


Fig.76. Chemical structure of Xylan.

For the present, there is less literature on fibrinolytics than antithrombosis drugs and platelet inhibitors, so the corresponding targets are fewer than those of antithrombosis drugs and platelet inhibitors. We summarized their respective targets and structural characteristics based on the reported literature, as shown in the Table 3.

Table 3. Fibrinolytic compounds and their respective targets

NO.	Compound name	Target	Structural characteristics
1	107	TXA ₂	diosgenin
2	108	TXA ₂	diosgenin
3	110	PAI-1	/
4	111	P-selectin	pyrido[3,4-b] indol

4. Conclusion

Uncontrolled thrombosis is the main cause for a variety of cardiovascular and cerebrovascular diseases, leading to the cardiovascular morbidity and mortality gradually increasing year by year [123]. Unexpectedly, even some persons occurred rare and severe blood clots after receiving the AstraZeneca COVID vaccine, leading to the suspending in many countries. Antithrombotic medicines are particularly significant due to their potency against thrombotic disorders to ameliorate popular conditions. Up to today, the primary treatment and prevent strategy have been use of the three categories: anticoagulants, platelet inhibitors and fibrinolytics, and the approved drugs like unfractionated heparin, low molecular weight heparins (LMWHs), warfarin, aspirin, ridogrel, dipyridamole, argatroban, dabigatran, streptokinase,

anistreplase and so on, they play important roles in clinical application but have insurmountable disadvantages such as the unexpected high risk of bleeding, a slow onset of action, a narrow therapeutic window, consequently, they require individualized medication and close monitoring. To circumvent these issues, extensive and deep investigations and tremendous endeavors have been made in exploiting novel antithrombotic agents in recent decades. Theoretically, any enzyme or receptor involved in the process of thrombosis can be used as the target of antithrombotic drugs [124], while the major researches focus on thrombin, various coagulation factors, GPIIb-IIIa receptor, ADP receptor and TXA₂ receptor [125] as discussed. With further studying on antithrombotic mechanism, discovery of more promising targets will bring along emergence of more anticoagulant agents with efficient, safe and orally bioavailable properties.

Declaration of competing interest

No personal or financial conflicts of interest have been found among all the authors.

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