

## Theme Issue Article

# From heparin to EP217609: The long way to a new pentasaccharide-based neutralisable anticoagulant with an unprecedented pharmacological profile

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### Summary

The elucidation of the structure of the antithrombin binding sequence in heparin has given a large impulse to the rational design of heparin related drugs. *De novo* chemical synthesis of the corresponding pentasaccharide as well as simplified analogues has provided very specific, antithrombin-mediated inhibitors of factor Xa with various pharmacokinetic profiles. Fondaparinux and idraparinux are examples of such compounds that have found clinical application as antithrombotics. Because of the very specific binding to antithrombin the pharmacokinetics of pentasaccharides can be predicted and transferred to other molecules covalently bound to them. The new chemical entities thus obtained display a wide array of antithrombotic activities, giving improved heparin molecules as well as new anticoagulants, devoid of the undesired side effects of heparin and with unprecedented

pharmacological profiles. In this context, a direct thrombin inhibitor was covalently coupled to a pentasaccharide by an inert spacer. This compound, EP42675 exerts antithrombin mediated anti-factor Xa activity together with direct thrombin inhibiting capacity. It displays favourable pharmacokinetics as imposed by the pentasaccharide. EP42675 was further modified by the introduction of a biotin moiety in its structure. The new entity obtained, EP217609 exerts the same pharmacological profile as EP42675 and it can be instantaneously neutralised by injection of avidin. Due to this unprecedented mechanism of anticoagulant activity and its ability to be neutralised, EP217609 deserves to be investigated in clinical settings where direct thrombin inhibition is required.

### Keywords

Antithrombin, coagulation inhibitors, drug design, heparin, thrombosis

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## Introduction

Heparin is a complex natural polysaccharide (1) that displays anticoagulant activity through activation of the plasma protein antithrombin (formerly called antithrombin III). Heparin has been used as an anticoagulant for about 70 years in a broad range of clinical thrombotic conditions (1–5). Standard heparin however has some considerable shortcomings such as poor bioavailability after subcutaneous administration, a poor pharmacokinetic profile and a potential to induce antibody associated thrombocytopenia due to interaction with platelet factor 4 (PF4). Better understanding of its action on the coagulation pathway led

to the development of the low-molecular-weight heparin (LMWH) class of drugs. LMWH showed improvement in the specificity of anticoagulant action and pharmacokinetic aspects. The developments in this area have recently been reviewed (6).

The emerging knowledge in the understanding of heparin's action has provided insight in the most critical chemical requirements for its anticoagulant activity. The detailed mechanistic understanding of this process has resulted in the total chemical synthesis of heparin-related products with highly specific pharmacodynamic profiles. The evolution from heparin to highly sophisticated anticoagulants (Fig. 1) is the topic of the present review.

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## Chemical and biochemical aspects of heparin

Standard heparin preparations are mixtures of glycosaminoglycan chains made of repeated disaccharide units (2-O-sulphate- $\alpha$ -L-iduronic acid coupled to 2-N-sulphate-6-O-sulphate- $\alpha$ -D-glucosamine) and have a mean molecular weight of approximately 15,000 daltons. A closer look at the structure of these chains reveals the presence, here and there, of other monosaccharide motives that play a crucial role in their anticoagulant activity. Thus, following the discovery of the role of antithrombin as heparin cofactor, affinity chromatography experiments allowed to fractionate heparin into a high-antithrombin-affinity fraction (1/3 of the preparation) and a low-antithrombin-affinity fraction (2/3 of the preparation). Only the former displayed anticoagulant properties. The major structural difference between the two fractions is a unique pentasaccharide sequence (the antithrombin binding site in heparin) that is responsible for the binding and the activation of antithrombin (Fig. 1). A detailed account of the work of the different groups that led to the discovery of this sequence has been published (7).

Latent antithrombin interacts very slowly with the serine proteases of the coagulation system. Heparin accelerates the antithrombin dependent inhibition of several activated coagulation factors: IIa/thrombin, IXa, Xa, XIa, XIIa. All these factors are inactivated by the formation of a 1:1 covalent complex with antithrombin. The effect of heparin during the inactivation of a serine protease can be divided into two cooperative effects: A conformational effect (change in conformation allowing antithrombin recognition by the enzyme) and a template effect (template onto which antithrombin and the enzyme will be positioned just before they interact). The antithrombin binding site in heparin is necessary to obtain the conformational effect allowing antithrombin binding and activation. Most remarkably, the conformational effect plays a major role in factor Xa inhibition, with a very minor contribution of the template effect in this case. This phenomenon forms the basis for the excellent factor Xa inhibitory properties of the antithrombin binding pentasaccharide (fondaparinux). The situation is completely different with the other coagulation factors, particularly thrombin, where both the conformational and the template effect are required to cause inhibition. In such cases a longer heparin fragment is involved (e.g. for thrombin a minimum chain length of ~18 monosaccharides is required).

Elucidating the mechanism of heparin's anticoagulant action required studies from several groups (8–16). Chemical synthesis of a number of heparin related oligosaccharides also contributed to this understanding by unambiguously confirming the role of many structural features present in heparin molecules. Most remarkably, the chemical synthesis of the pentasaccharide sequence responsible for antithrombin binding confirmed unambiguously that this fragment selectivity enhances the inactivation of factor Xa, but not of thrombin (17, 18). All these efforts paved the way towards synthetic drugs with tailor-made anticoagulant profiles (for review see [19, 20]).

## Pentasaccharide-based antithrombotics selectively inhibiting factor Xa: chemistry and pharmacology

From the standpoint of drug design, the major breakthrough in the history of heparin was certainly the discovery that the pentasaccharide representing the antithrombin binding site was able to selectively inhibit factor Xa (17, 18). Indeed, the antithrombotic potential of factor Xa inhibitors was a matter of debate in the 80s and it was only when selective factor Xa inhibitors could be obtained that this potential could be unanimously acknowledged. On top of this, the total chemical synthesis of such compounds, although not trivial, would allow to rationally design pentasaccharide-based new antithrombotics.

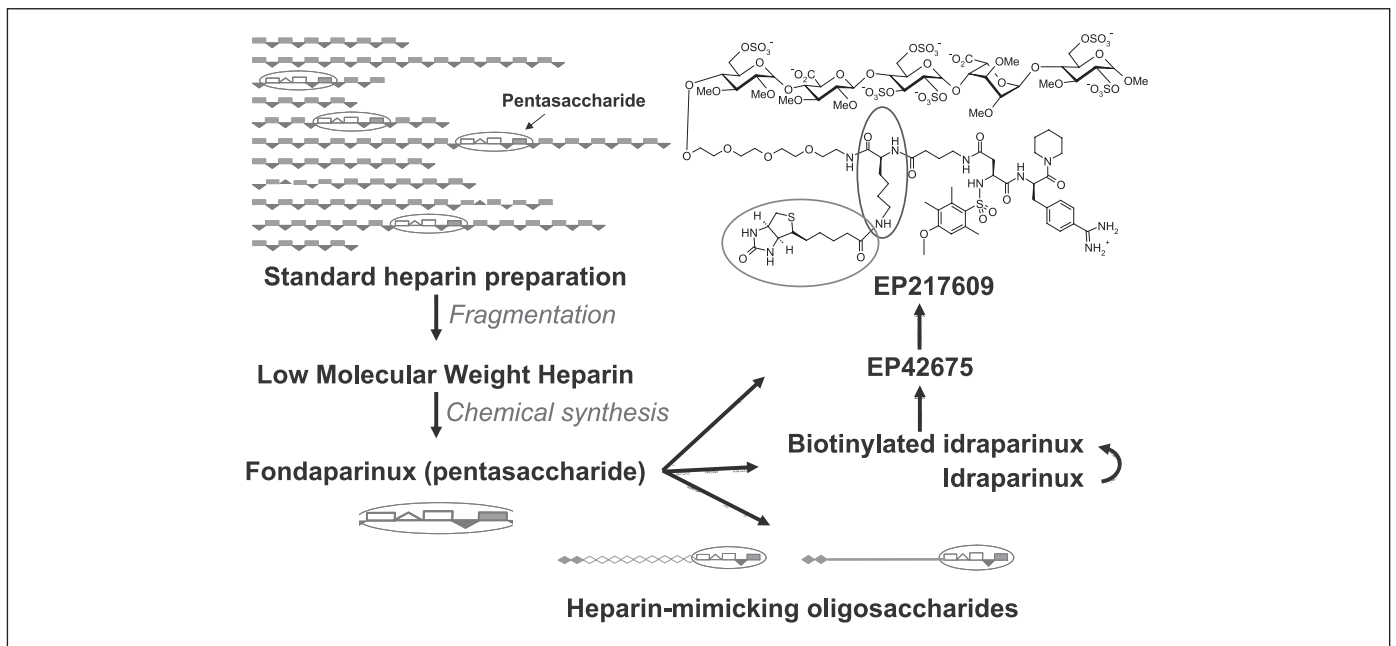
To this end, in the first place the essential structural features in the „natural“ pentasaccharide have been identified (19). This was executed by focused chemical synthesis of pentasaccharide-analogues in which selected constituents were omitted or replaced. These compounds were tested with respect to binding to antithrombin, factor Xa inactivation, antithrombotic effect in experimental thrombosis models and pharmacokinetic profile.

In parallel, to simplify the production of such potential drugs, easier to synthesise pentasaccharide analogues displaying similar or enhanced pharmacological activity were produced (19). More than a hundred compounds have been obtained and tested. The general pattern that emerged was that all *N*-sulfate groups could be replaced by *O*-sulfate groups and all hydroxyl groups could either be *O*-sulfated or *O*-methylated without loss of activity. Depending on the presence or absence of particular substituents in the essential pentasaccharide structure, compounds with varying binding capacity to antithrombin could be obtained. Binding could be markedly increased through replacement of a 3-OH group by an *O*-sulfate group.

Pharmacological testing revealed that all compounds with accelerating effect on the antithrombin mediated inactivation of factor Xa (anti-Xa activity) exert antithrombotic activity in various experimental thrombosis models using different animal species (21, 22), the antithrombotic activity being related to their anti-Xa activity. It was also noticed that the duration of action of anti-Xa and antithrombotic activity was related to the binding affinity to antithrombin (23). A number of compounds were selected for further clinical evaluation selection criteria being feasibility for large scale chemical synthesis and anticipated duration of action.

Two compounds out of this series, fondaparinux and idraparinux have been developed further for application in man. Fondaparinux is practically identical to the antithrombin binding pentasaccharide sequence in heparin. It enhances the antithrombin mediated factor Xa inactivation approximately 300-fold. This compound is now a drug administered by the subcutaneous route. Its absorption is complete, rapid and independent of the dose. Its elimination half-life in man is approximately 17 hours. It exerts efficacy after daily subcutaneous administration in various thrombotic conditions (24–26).

The major chemical difference between idraparinux and fondaparinux is the presence of an additional 3-*O*-sulfate group in the glucose moiety at the reducing end of idraparinux that



**Figure 1: From heparin to synthetic heparin-derived anti-thrombotics.** A standard heparin preparation (top left) is a mixture of polysaccharidic chains containing repeated disaccharide units (glucosamine/rectangle-uronic acid/triangle). These chains vary in length and in structure. The ovals indicate the presence in some of these chains of the pentasaccharide sequence representing the antithrombin binding domain (ABD). Low Molecular Weight Heparins can be obtained from standard heparin after enzymatic or chemical fragmentation. The ABD can be reproduced by total chemical synthesis to generate the pentasaccharide fondaparinux. Idraparinux is an analogue of fondaparinux endowed with

very high affinity for antithrombin. Other antithrombotic oligosaccharides able to catalyse factor Xa and thrombin inhibition by antithrombin were also obtained by total chemical synthesis (bottom right). More recently a biotin entity was introduced on idraparinux to allow its neutralisation by injection of avidin. Finally even more sophisticated anticoagulant like EP42675, and particularly EP217609 the neutralisable version of EP42675, were synthesised by combining a fondaparinux analogue and a direct thrombin inhibitor. The structure of EP42675 can be deduced from that of EP217609 by deletion of the biotine –red oval- and lysine –blue oval- residues.

causes a significantly stronger binding to antithrombin. The potency to inactivate factor Xa *via* antithrombin and the half-life of elimination is therefore markedly increased. The half-life of the drug after subcutaneous administration is approximately 120 hours after one subcutaneous administration and increases to 60 days after 6–12 months one weekly subcutaneous administration (27, 28). It needs to be administered only once a week and shows antithrombotic efficacy in man (29, 30).

### Oligosaccharide-based antithrombotics mimicking the anticoagulant activity of heparin: chemistry and pharmacology

Following the successful development of pentasaccharides with specific anti-factor Xa activity the rational design of heparin mimetics able to inhibit thrombin on top of factor Xa was pursued. The development of such compounds could be of clinical importance since the precise pharmacological profile of the ideal anticoagulant drug still remains to be determined. The targeted compounds would have a perfectly defined molecular composition and therefore would differ from heparin and LMWHs. They would also be devoid of the non-specific interactions developed by heparins of natural origin, such as neutralisation by platelet factor 4 (PF4) and associated induction of thrombocytopenia.

Heparin induced thrombocytopenia (HIT) is caused by heparin dependent antibodies of the IgG-type, that are directed against a molecular complex formed by heparin and platelet factor 4 (PF4). These IgG immune complexes bind to the FcγIIa receptors on platelets, resulting in platelet aggregation and eventually the occurrence of thrombocytopenia (31).

As indicated above, antithrombin-mediated inactivation of thrombin requires a heparin fragment containing at least 18 monosaccharides. A next step was thus to identify the position of the antithrombin binding domain (ABD) in the ~18 oligosaccharide. Molecular modelling provided an answer to this question that was confirmed by synthesis and testing of active and inactive molecules (reviewed in 19 and 20). Two different approaches were then followed for the synthesis of compounds displaying both factor Xa and thrombin inhibitory properties (Fig. 1).

In the first approach a neutral non-carbohydrate spacer was used to connect the antithrombin binding domain (ABD) through its non-reducing end to another negatively charged (sulphated) oligosaccharide representing the thrombin binding domain (TBD). The challenge was to find out the optimal spacer-length to connect the ABD with the TBD and the optimal charge density of the TBD. Out of the series of compounds obtained comprising idraparinux as the ABD, a heptasulfated cellobiose disaccharide as the TBD and a 53 atoms long polyethylene glycol

spacer in between was pharmacologically evaluated (32). *In vitro* the compound showed high anti-Xa activity and moderate thrombin neutralising activity. It did not show cross-reactivity with HIT-antibodies and was not neutralised by PF4. *In vivo* this compound was equipotent with fondaparinux in venous experimental thrombosis models but showed a superior activity in arterial experimental thrombosis models, thus exemplifying the added benefit of combining two pharmacological complementary mechanistic actions into one synthetic molecule.

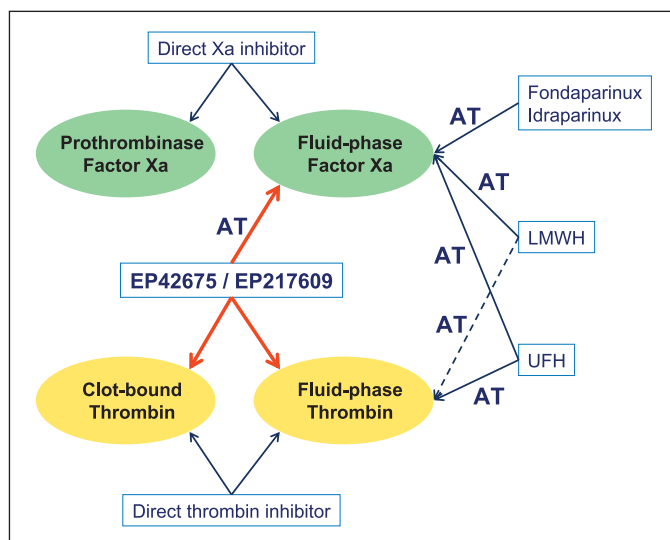
In the second approach, fully synthetic oligosaccharides long enough to mimic the heparin 18-mer template were used. Among them a fully synthetic hexadecasaccharide (16-mer) displayed interesting pharmacological properties and was selected for further development (33). In this compound a methylated nonasaccharide was used to bridge the ABD pentasaccharide to a TBD disaccharide (Fig. 1). As expected this compound catalysed inactivation of factor Xa and thrombin *via* antithrombin. It did not activate blood platelets and did not bind to PF4. It showed a favourable activity in experimental thrombosis models, being potent against experimental venous thrombosis and arterial thrombosis models.

While developing oligosaccharides consisting of ABD and TBD moieties connected by spacers it became apparent that due to its highly specific binding characteristics ABD could serve as a carrier for other types of anticoagulants and that completely new drugs could be designed (see also the use of ABD for the design of long-lasting insulin, 34).

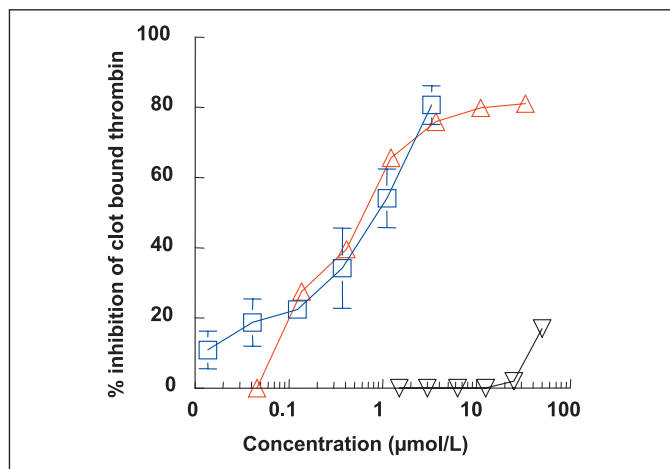
## Pentasaccharide-coupled direct thrombin inhibitor: Unprecedented pharmacological profile

Heparin and LMWHs are still the reference antithrombotic drugs. However one of their shortcomings is probably related to their inability to inhibit clot-bound thrombin, and there is a need for effective suppression of clot/thrombus associated thrombin generation in several acute clinical situations, e.g. acute coronary syndromes (ACS). Direct thrombin inhibitors (DTIs) represent a potential solution in this situation but previous clinical trials with DTI's have failed, possibly because of insufficient duration of action of the anticoagulant used (35, 36). Therefore the most important features of a suitable anticoagulant for acute situations would be: i) proven efficacy to inhibit clot associated procoagulant activity, and ii) favourable pharmacokinetic profile ensuring prolonged anticoagulant coverage with improved control over its therapeutic window. For all these reasons, coupling an antithrombin binding pentasaccharide to a direct thrombin inhibitor (37, 38) is an appealing design for an antithrombotic drug to be used in conditions with persistent thrombin generation. This idea is further supported by the results of recent clinical trials with fondaparinux (a pentasaccharide) in ACS that seem to indicate that the only drawback of the compound lies in its inability to inhibit thrombus formation on the catheter used during the procedure.

The synthesis of a compound consisting of a fondaparinux analogue conjugated *via* a spacer to an  $\alpha$ -NAPAP derivative (39) has been realised. The  $\alpha$ -NAPAP derivative inhibits thrombin in a competitive manner ( $K_i$  15 nM) by binding to exosite 1 and/or



**Figure 2: Inhibition of factor Xa and thrombin by different types of anticoagulant agent.** This scheme shows that EP42675 and EP217609 display a unique mechanism of action. EP42675 and EP217609 combine direct inhibition of thrombin (fluid phase or clot bound) with indirect inhibition of factor Xa, thus mimicking DTIs and part of the activity of heparin. The presence of "AT" next to the arrow indicates that the compound is active through antithrombin activation. AT: Antithrombin. UFH, unfractionated heparin; LMWH, low-molecular-weight heparin.

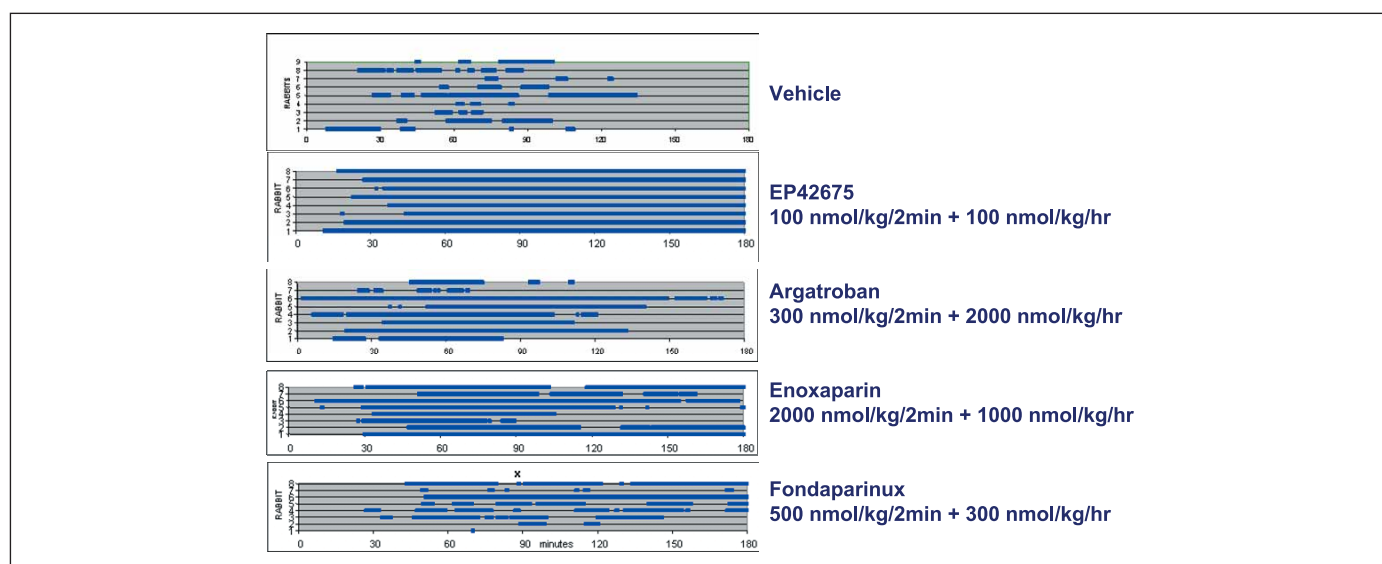


**Figure 3: Inhibition of clot-bound thrombin.** EP42675 (squares), argatroban (triangle pointing up) and heparin (triangle pointing down) were compared. In contrast with heparin EP42675 inhibits clot bound thrombin. The assay was performed according to Berry et al., *Thromb Haemost* 1994; 72: 381–386.

the active site of thrombin. The potential advantage of direct thrombin inhibitors lies in their capacity to inhibit both fibrin-bound as well as fluid phase thrombin. On the other hand, DTIs in general have the disadvantage of short plasma half-lives (40).

The unique (Fig. 2) pharmacological profile of EP42675 (41), shows the following characteristics:

- It inhibits factor Xa in the presence of antithrombin.



**Figure 4: Antithrombotic effect of various anticoagulants in the rabbit carotid artery model.** A thrombus is formed in the carotid artery, then lysed using streptokinase in the presence of the tested agent. Blood flow is monitored. Each line represents one animal. A blue bar indicates that the vessel is patent while a dark line denotes an occluded vessel. Thrombolysis was started by a bolus injection of 30,000 U/kg Streptokinase (Kabikinase®) IV and 1200 U/kg/min maintenance infusion during 30 min in conjunction with the tested compound. EP42675 was administered in a bolus of 100 nmol/kg/min (2 min) followed by a main-

tenance dose of 100 nmol/kg during 60 minutes. Blood flow was measured until 2 hours after cessation of the infusion of the tested compound. Argatroban was administered with a bolus of 300 nmol/kg/min (2 min) and a maintenance dose of 2000 nmol/kg/min during 1 hour. Fondaparinux was administered with a bolus of 500 nmol/kg/min (2 min) and a maintenance dose of 300 nmol/kg during 1 hour. Enoxaparin was administered with a bolus of 2000 nmol/kg/min (2 min) and a maintenance dose of 1,000 nmol/kg/min during 1 hour.

- It has a direct, antithrombin-independent inhibiting effect on free fluid thrombin as well as clot-bound thrombin. It inhibits clot-bound thrombin generation to a similar extent as argatroban, whereas unfractionated heparin (UFH) fails to suppress clot-bound thrombin generation (Fig. 3).
- It exerts a dose dependent effect on overall coagulation tests (APTT, PT, TCT). These effects are mainly related to the DTI part of the molecule since fondaparinux alone has no effect on these parameters.
- It inhibits thrombin induced platelet aggregation, but has no effect on adenosine diphosphate (ADP) or collagen induced aggregation.
- It does not cross-react with PF4 antibodies.

EP42675 is stable in plasma as shown by the thrombin inhibitory activity and the factor Xa inhibitory activity disappearing at the same rate after either intravenous or subcutaneous adminis-

**Table 1: Inhibition of factor Xa and thrombin by EP42675 and EP217609.** The compounds display identical  $IC_{50}$  indicating that the introduction of a biotin moiety (EP217609, see Fig. 1) neither interferes with the activation of antithrombin by the pentasaccharide part, nor with the inhibitory properties of the direct thrombin inhibitor.  $IC_{50}$ : Mean of two experiments performed in triplicate  $\pm$  SD.

	EP42675	EP217609
Anti-Xa ( $IC_{50}$ , nM)	12.83 $\pm$ 2.33	11.50 $\pm$ 1.26
Anti-IIa ( $IC_{50}$ , nM)	13.65 $\pm$ 2.33	11.48 $\pm$ 1.97

tration (41). The half-life of EP42675 in rats is approx. 3 hours, whereas the half-life of the non-coupled  $\alpha$ -NAPAP derivative is 10-fold shorter. The bioavailability after subcutaneous administration is 100%.

*In vivo*, the effect on thrombosis prevention was demonstrated in four experimental thrombosis models representing venous as well as arterial thrombus formation (41). In all these models EP42675 is, on a molar basis, more potent than UFH, fondaparinux and argatroban. EP42675 was also evaluated in an experimental thrombolysis model in comparison with UFH, argatroban and fondaparinux. In this model the antithrombotic potency of the drugs was evaluated both during thrombolysis and after termination of the thrombolytic therapy. EP42675 is fully preventive against thrombotic reocclusion as opposed to the other treatments (Fig. 4).

EP42675 has an enhancing effect on surgically induced bleeding, this effect being significant at doses which are approximately 10-fold higher than the  $ED_{50}$  in the thrombosis models. With respect to safety efficacy ratios that can be calculated from the *in vivo* experiments performed, EP42675 compares favourably with other antithrombotic compounds evaluated under similar conditions.

### Biotinylated anticoagulant neutralised by avidin

The use of anticoagulants can result in haemorrhagic adverse events and the availability of an antidote is highly suitable. An antidote can also be useful in case an anticoagulated patient needs to be urgently operated. To this end a biotin moiety has

been introduced on idraparinux, a long lasting anti-factor Xa pentasaccharide currently in clinical development (42). The resulting compound, SSR126517E is immediately and definitively removed from plasma after injection of avidin, a very short half-life protein that displays very high affinity for biotin ( $K_d$  in the order of  $10^{-15}$  M).

To make EP42675 an even more attractive anticoagulant drug particularly useful in acute clinical situations where there is a medical need for a potent fast-on/fast-off anticoagulant, a similar principle was pursued to incorporate the possibility for neutralising the compounds anticoagulant activities. Without having any precedence for successful neutralisation of a dual acting pharmaceutical entity by avidin, a biotin entity was covalently linked to the spacer between the pentasaccharide part and the DTI part of the molecule (43). The resulting compound, EP217609 (Fig. 1), demonstrates an anticoagulant and antithrombotic profile which is identical to that of EP42675 (Table 1). The elimination half-life, as determined by the residual anti-factor Xa, and thrombin inhibitory activities are also identical to that of EP42675 (Fig. 5). More importantly, *in vivo* administration of avidin to animals treated with EP217609 led to immediate disappearance of both anti-Xa and thrombin inhibiting activities (Fig. 6). Thus, in this case not only the antithrombin mediated anti-Xa inhibitory activity but *simultaneously* the DTI component is neutralised. The specific mode of action underlying this instantaneous eradication of anticoagulant activity was corroborated by the fact that the dual inhibitory activity observed in EP42675 treated animals was unaffected by treatment with avidin.

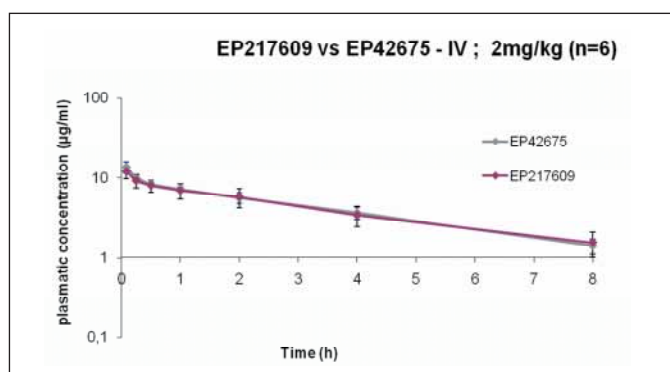
In summary, EP217609 is an effective antithrombotic compound with an unprecedented pharmacological profile (anti-thrombin-mediated factor Xa inhibition and direct thrombin inhibition) that can be immediately neutralised by the injection of a specific antidote.

## Concluding remarks

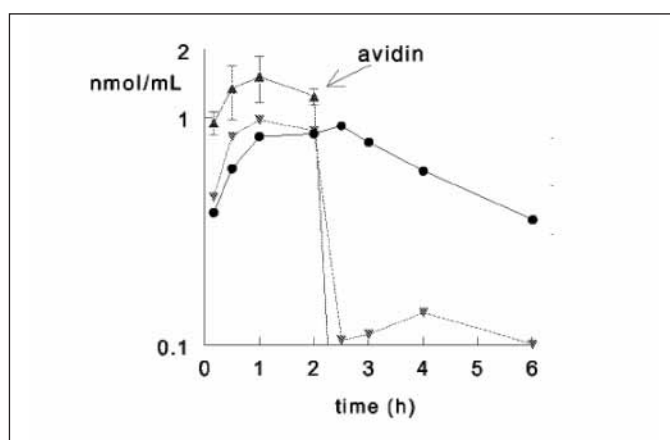
The elucidation of the molecular mechanism of action of the established anticoagulant heparin paved the way for the development of a new generation of pentasaccharide based anticoagulant agents acting through activation of antithrombin leading to specific inhibition of coagulation factor Xa. The clinical efficacy of fondaparinux and idraparinux has demonstrated the validity of this approach. In the present article we have reported how medicinal chemistry applied to similar pentasaccharides can be used to produce new chemical entities with which several shortcomings of heparin and other anticoagulants can be circumvented. Clinical trials will tell if the „super-heparin“ EP217609 described here is safe and effective up to the wishes of the scientists who rationally designed it.

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**Figure 5: Comparison of the pharmacokinetics of EP42675 and EP217609 in rat after IV administration.** The deduced elimination half-lives were 2.7 h and 2.9 h for EP42675 and EP217609, respectively.



**Figure 6: EP217609 can be neutralised by injection of avidin.** EP42675 and EP217609 were injected to rats (n=3) and blood was punctured at various time points. The anticoagulant activity was monitored using factor Xa and thrombin assays. Injection of avidin resulted in a complete disappearance of EP217609 (anti-Xa activity: Triangle pointing down; anti-IIa activity: Triangle pointing up) while it had no effect on EP42675 (anti-Xa activity: Circles).

## Abbreviations

ABD, Antithrombin binding domain; ACS, Acute coronary syndrome; ADP, Adenosine diphosphate, APTT, Activated partial thromboplastin time; AT, Antithrombin; DTI, Direct thrombin inhibitor; HIT, Heparin Induced Thrombocytopenia; LMWH, Low Molecular Weight Heparin; NAPAP, [N<sup>a</sup>-(2-naphthyl-sulfonyl-glycyl)-DL-p-amidinophenylalanyl-piperidine]; PF4, Platelet factor 4; PTT, Prothrombin time; TBD, Thrombin binding domain; TCT, Thrombin clotting time; UFH, Unfractionated heparin; VTE, Venous thromboembolism.

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