Evaluating the effects of sugammadex on coagulation in humans: reversed translational research to unravel off-target pharmacology

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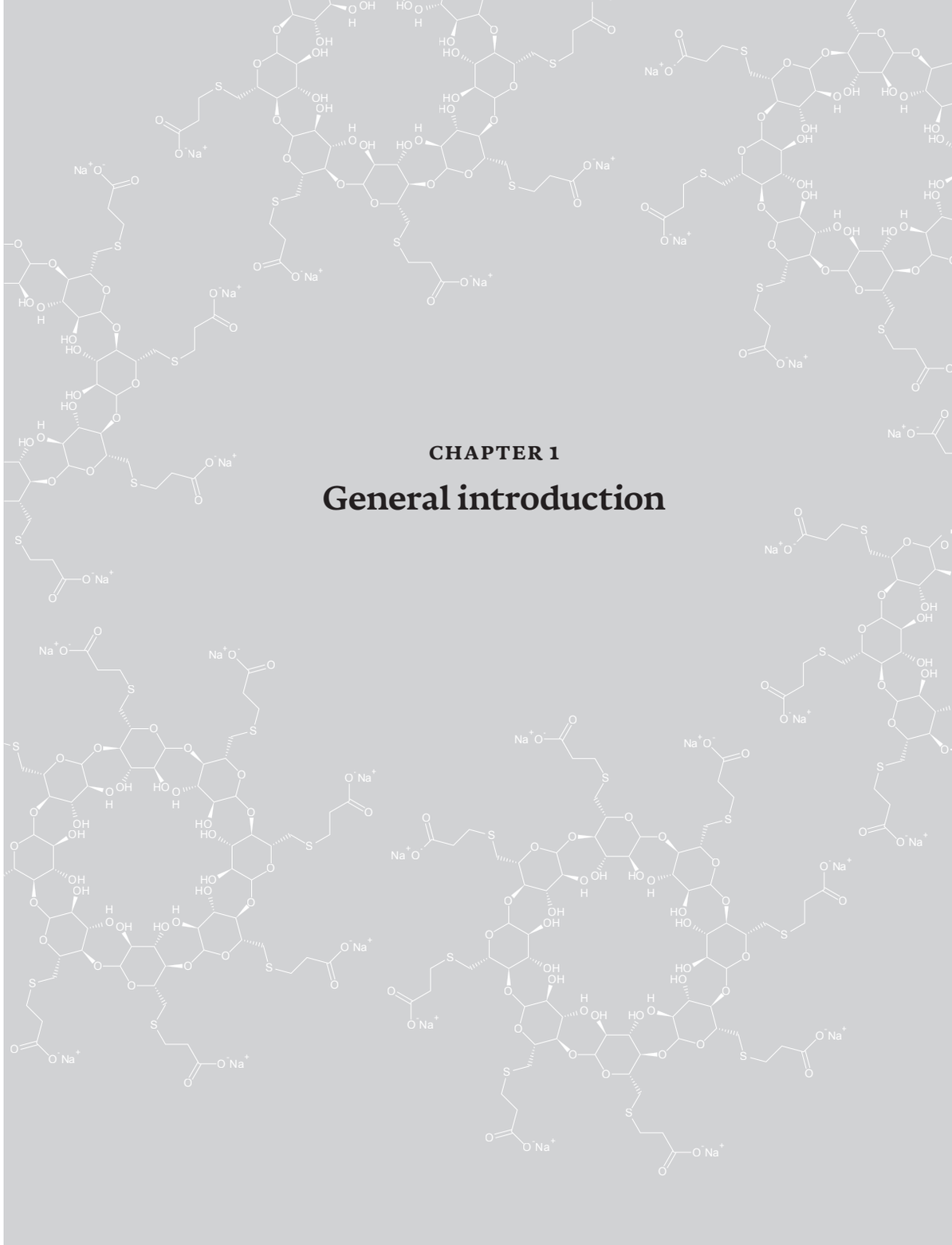


CHAPTER 1

General introduction

Neuromuscular blockade

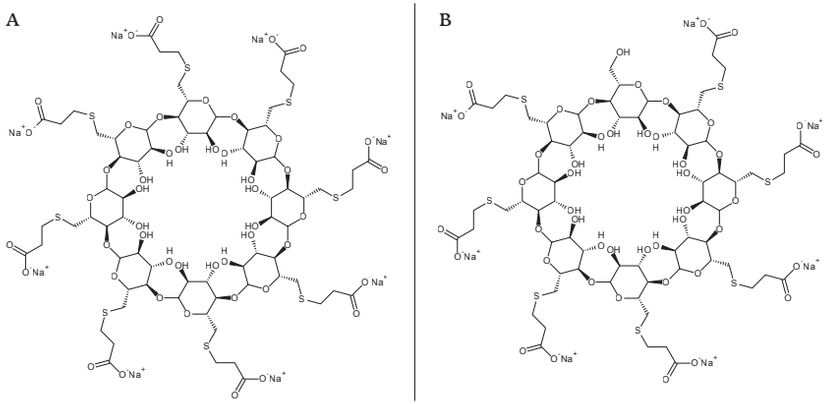
Neuromuscular blocking agents (NMBAs) are used in general anesthesia to facilitate tracheal intubation and achieve skeletal muscle relaxation during surgery.[1] The first NMBAs emerged mid-20th century and originate from curare, an extract of various plants used as paralyzing poison on arrow tips by South American Indians for centuries.[2] NMBAs revolutionized the concept of anesthesia into a triad of narcosis, analgesia and muscle relaxation, known as the 'Liverpool technique'.[3] There are two classes of NMBAs that are known to interfere with the action of the neurotransmitter acetylcholine at the postsynaptic nicotinic receptors in the neuromuscular junction: depolarizing and non-depolarizing NMBAs. Binding of acetylcholine to its receptors causes ion channels to open resulting in depolarization of the motor end plate and subsequent muscle contraction. Depolarizing NMBAs act as acetylcholine agonists but are resistant to metabolism by acetylcholinesterase resulting in prolonged depolarization of the motor end plate, while non-depolarizing NMBAs act as competitive antagonists.[4] Suxamethonium (succinylcholine) is currently the only widely available depolarizing NMBA[2,3] and is metabolized by butyrylcholinesterase (pseudocholinesterase) causing a short-term neuromuscular blockade.[5] Non-depolarizing NMBAs is a broader class with as main lead chemical structures benzylisoquinolinium and aminosteroids.[4] In general, non-depolarizing NMBAs act longer than suxamethonium. Their introduction engendered the development of reversal agents,[6] especially for situations when immediate reversal is needed because of difficulties in airway management.[7] Traditionally, reversal was achieved by administration of an acetylcholinesterase inhibitor (anticholinesterase) such as neostigmine. The reversal strategy is to achieve sufficient accumulation of acetylcholine to competitively displace the non-depolarizing NMBA.[4] Anticholinesterases can also affect muscarinic acetylcholine receptors causing side effects such as nausea and bradycardia.[8] Their application is further limited to blockades with residual neuromuscular activity, and unsuitable for immediate reversal of deep/profound blockades.[9] In addition, residual or recurrent paralysis, associated with post-operative complications such as respiratory failure, can still occur.[10,11]

These shortcomings can be overcome by another reversal strategy. Instead of increasing the concentration of acetylcholine, neuromuscular function can also be restored by decreasing the concentration of the NMBA.[7] This constituted a new class of selective relaxant binding agents, of which sugammadex is the first agent[12] and described in greater detail below.

Sugammadex

The discovery of cyclodextrins as potential reversal agent of steroidal NMBAs was a serendipitous finding. Cyclodextrins, cyclic oligosaccharides composed out of 6, 7 or 8 glucosyl-units (α-, β- and γ-cyclodextrin, respectively) framing a hydrophilic truncated cone with a hydrophobic interior, were applied for the enhancement of water-solubility and bioavailability of pharmacologically active substances. In 1997, Anton Bom and his fellow researchers at Organon Laboratories in Newhouse, Scotland, successfully attempted to improve the solubility of rocuronium (ORG 9426) by using cyclodextrins. They discovered that the sequestering of rocuronium by cyclodextrin rendered rocuronium unable to exert its pharmacological effect. This was explained by the affinity of the aminosteroidal structure of rocuronium for cyclodextrin's cavity.[13] This affinity depended on the cavity size, with γ-cyclodextrin having the highest potency.[9] The γ-cyclodextrin structure was further optimized to lower its association constant (K_a) for rocuronium by adding side chains to extend the cavity, enabling complete encapsulation.[13] Further, addition of negatively charged end-groups prevented that the side chains would close off the cavity and increased the affinity by interaction of these end-groups with the positively charged quaternary nitrogen of rocuronium.[9] One of the most potent modified γ-cyclodextrins was ORG 25969. This compound is currently known as sugammadex,[14] which is an abbreviation of sugar (su) and the molecule structure γ-cyclodextrin (gammadex).[12] During the synthesis of sugammadex, a byproduct called ORG 48302 is formed that has one carboxyl thioether group less than sugammadex, but a pharmacological profile similar to sugammadex, with approximately 50% lower affinity for rocuronium and vecuronium (ORG NC45). The drug substance sugammadex contains up to 7% ORG 48302.[15] Since its discovery, the ownership of sugammadex shifted from Organon to Schering-Plough in 2007. Two years later Schering-Plough merged with Merck & Co. (doing business as Merck Sharp & Dohme outside the United States of America (USA) and Canada), the current owner of sugammadex. The structure formulas of ORG 25969 and ORG 48302 are provided in Figure 1.

Figure 1 Structure formula of (A) ORG 25969 and (B) ORG 48302, image courtesy of Wikimedia Commons and Folkert A. van Meurs, respectively.

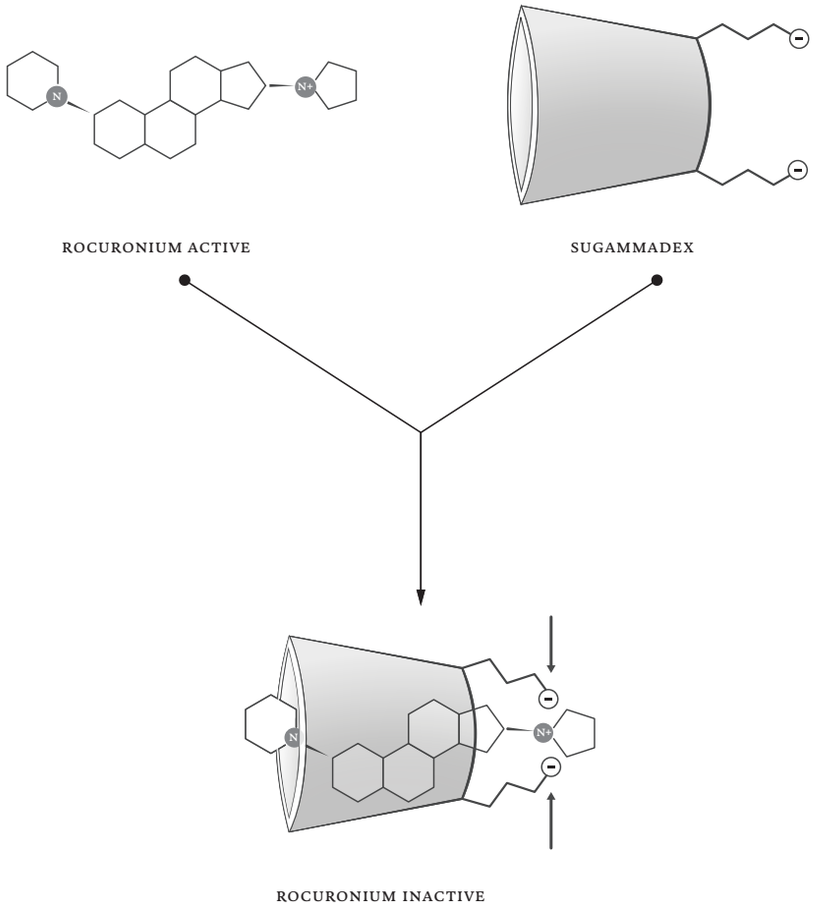


Sugammadex has a very high affinity for the steroidal NMBAs rocuronium and vecuronium, with a 2.5-fold difference in favor of the former,[13] and a moderate affinity for pancuronium (ORG NA97), but lacks affinity for the nonsteroidal NMBAs.[8] Sugammadex forms complexes with steroidal NMBAs with a 1:1 stoichiometry. This encapsulation process (see Figure 2) occurs in plasma upon intravenous administration of sugammadex and prompts NMBA molecules to navigate from their site of action at nicotinic receptors in the neuromuscular junction towards plasma in order to reestablish the concentration equilibrium, thereby restoring the muscle function.[16]

In clinical practice, sugammadex demonstrated a dose-dependent, fast, effective and complete reversal of any degree of neuromuscular blockade induced by rocuronium or vecuronium.[1,8,17–21] When an adequate dose of sugammadex is administered, both rocuronium- and vecuronium-induced blockades are reversed in minutes and without post-operative residual or recurrent neuromuscular blockade.[22–24] Sugammadex is well tolerated and free from muscarinic side effects.[16] Intravenous bolus doses of sugammadex in the therapeutic range (up to 16 mg/kg) demonstrated linear kinetics. The elimination half-life of sugammadex is about 2 hours.[25] Sugammadex is primarily cleared via the kidney, with minimal or no metabolism,[26] with a clearance rate similar to the glomerular filtration rate in healthy subjects.[27]

Sugammadex (trade name Bridion®) is currently registered in more than 80 countries.[19] For adults, a dose of 2 and 4 mg/kg sugammadex is indicated for routine reversal of moderate and deep blockade, respectively, and 16 mg/kg sugammadex for reversal 3 minutes after an intubating dose of 1.2 mg/kg rocuronium.[19] In some countries, a dose of 2 mg/kg for the routine reversal of rocuronium-induced blockade in children and adolescents aged 2 to 17 years is supported.[28]

Figure 2 Encapsulation process of a rocuronium molecule by a sugammadex molecule; image courtesy of Folkert A. van Meurs. Arrows at rocuronium inactive indicate the electrostatic interaction between the positively charged quaternary nitrogen of rocuronium and the negatively charged end-groups groups of sugammadex.



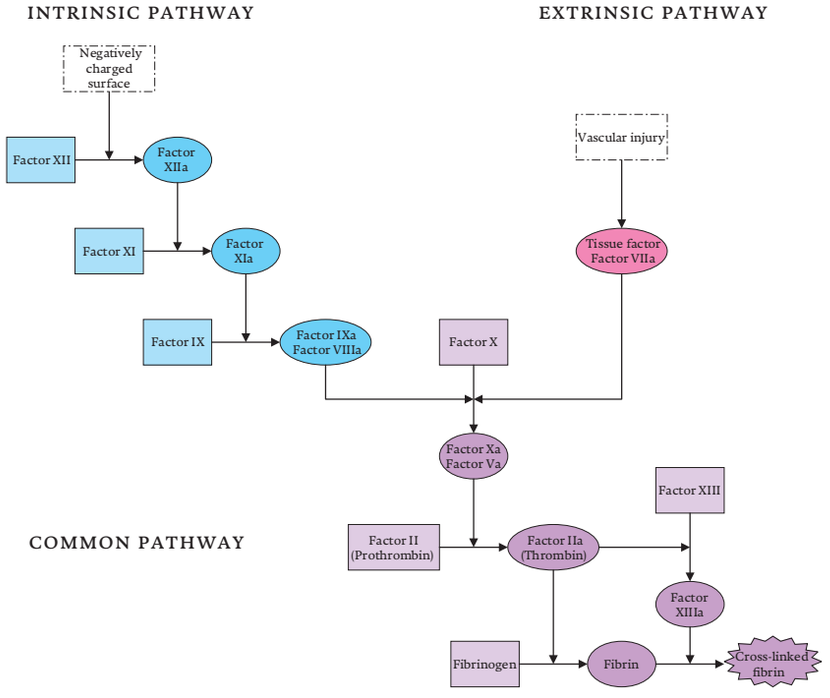
Before sugammadex became available for clinical use, its registration dossier was subject to regulatory review and approval. Potential safety concerns raised by regulatory agencies included the effect of sugammadex on coagulation and the clinical relevance thereof. This is described in more detail following a brief overview of coagulation.

Coagulation

Hemostasis is the process of blood clot formation at the site of vessel injury to stop bleeding while maintaining blood in a fluid state within the circulatory system. Upon vascular injury, platelets adhere to exposed collagen and then to each other to form a soft aggregate plug, mediated by von Willebrand factor. Platelet adhesion triggers activation pathways involving the release of platelet constituents from granules that amplify thrombus formation via positive feedback loops and control the growth of the plug. In addition, vasoconstrictors are released that activate vascular smooth muscles cells to reduce blood flow. Damaged endothelium also directly stimulates vasoconstriction. These processes contribute to the initial platelet plug formation, the so-called primary hemostasis, followed by secondary hemostasis leading to a stable, insoluble fibrin-rich plug by activated coagulation factors.[29–31] Coagulation factors, circulating mainly in an inactive form, such as zymogen serine proteases, are part of a cascade of enzymatic reactions in which each factor is activated by its preceding factor and catalyzes the activation of its downstream factor leading to strong amplification of the reaction. Coagulation factors have generally assigned Roman numerals, with a suffix 'a' to indicate the active form. The coagulation cascade has been traditionally classified into an intrinsic and extrinsic pathway, both of which converge on the common pathway at the level of factor X activation. This concept is known as the cascade or waterfall model of coagulation and is depicted in Figure 3.

The extrinsic and intrinsic pathway are initiated by exposed subendothelial tissue factor (TF) or negatively charged surfaces, respectively. Along the extrinsic pathway, TF and factor VIIa contribute to the formation of the extrinsic activator complex of factor X (extrinsic tenase). The intrinsic pathway (also known as contact pathway) starts with activation of factor XII, followed by activation of factor XI and subsequent factor IX. Factor IXa and VIIIa are instrumental to intrinsic tenase. Upon activation of factor X by the extrinsic or intrinsic tenase, factor Xa assembles with factor Va into the prothrombinase complex that cleaves factor II (prothrombin) yielding factor IIa (thrombin). Thrombin mediates the conversion of the soluble fibrinogen molecule into insoluble fibrin monomer, which polymerizes to form fibrin strands. The bonds between the strands are initially weak, but strengthened by thrombin-activated factor XIII. Several coagulation steps such as intrinsic tenase and prothrombinase complex activities are catalyzed by the cofactors calcium and/or negatively charged phospholipids. The coagulation pathway is tightly regulated by endogenous inhibitors, for instance by tissue factor pathway inhibitor (TFPI), antithrombin, and protein C.[31–33]

Figure 3 The cascade model of coagulation. Coagulation factors are part of a cascade of enzymatic reactions in which each factor is activated by its preceding factor. The cascade is initiated by two distinct pathways, the intrinsic and the extrinsic pathway, which converge on the common pathway at the level of factor X activation. Rectangular shapes and ellipses represent coagulation factors in inactive and active form, respectively.



The intrinsic and extrinsic pathways of the coagulation cascade are evaluated by the routine coagulation screening tests. Clotting time via the intrinsic pathway is assessed with activated partial thromboplastin time (APTT). The test is started by adding calcium, clotting active-phospholipids and a highly charged surface activator such as kaolin or silica to a plasma sample. Prothrombin time (PT) evaluates clotting time along the extrinsic pathway and is initiated by adding TF, phospholipids and calcium ions to plasma. The PT is highly sensitive to the type and batch of TF used in the assay. Standardization is achieved by using the international normalized ratio (INR), which expresses the PT of the test sample relative to a control sample, accounting for the sensitivity of the used TF.[30,31,34]

The classical view of coagulation as a cascade of reactions initiated by two distinct pathways leading to the common pathway serves the understanding of coagulation *in vitro* as assessed with APTT and PT, but it does not explain several clinical observations. For instance, individuals with factor XII deficiency do not show increased bleeding tendency despite a markedly prolonged APTT. In contrast, deficiency of factors VIII and IX causes bleeding disorders hemophilia A and B, respectively, demonstrating that both intrinsic pathway factors are required for adequate fibrin formation despite the presence of an intact extrinsic pathway. Hence the intrinsic and extrinsic pathways are more interdependent than suggested by the cascade model. In addition, the central role in the cascade model has been attributed to coagulation factors while cells merely provide a phospholipid surface. In physiological conditions, the cellular component is more essential as coagulation reactions appear to be localized to specific cell surfaces. A cell-based model has been developed as a refinement of the cascade model to more accurately reflect coagulation *in vivo*[35] and is illustrated in Figure 4 and Figure 5.

The cell-based model of coagulation consists of three overlapping phases called initiation, amplification and propagation (Figure 4). The initiation phase occurs on TF-bearing cell surfaces upon exposure of TF to plasma following vascular damage. This leads to the generation of trace amounts of thrombin via the extrinsic and common pathways. In addition, the complex of TF and factor VIIa activates factor IX. Any factor Xa dissociating from TF-bearing cells is rapidly inhibited by TFPI or antithrombin (Figure 5). TFPI inhibits factor Xa directly and inactivates extrinsic tenase in a factor Xa dependent manner, while antithrombin mainly inhibits factor Xa and thrombin. During the amplification phase, the initially generated thrombin moves

Figure 4 The cell-based model of coagulation. Coagulation is structured in three phases: initiation, amplification and propagation. Initiation occurs on tissue factor (TF) bearing cells, amplification on platelets as these become activated and propagation on activated platelets. Following TF exposure upon vascular injury, coagulation is initiated and trace amounts of thrombin are generated. The initial generated thrombin diffuses to nearby platelets where it amplifies coagulation by activation of platelets and several coagulation factors. This sets the stage for propagation leading to a burst of thrombin generation and formation of a stable fibrin clot. Rectangular shapes and ellipses represent coagulation factors in inactive and active form, respectively. Dashed arrows represent diffusion of coagulation factors from one type of cell surface to another.

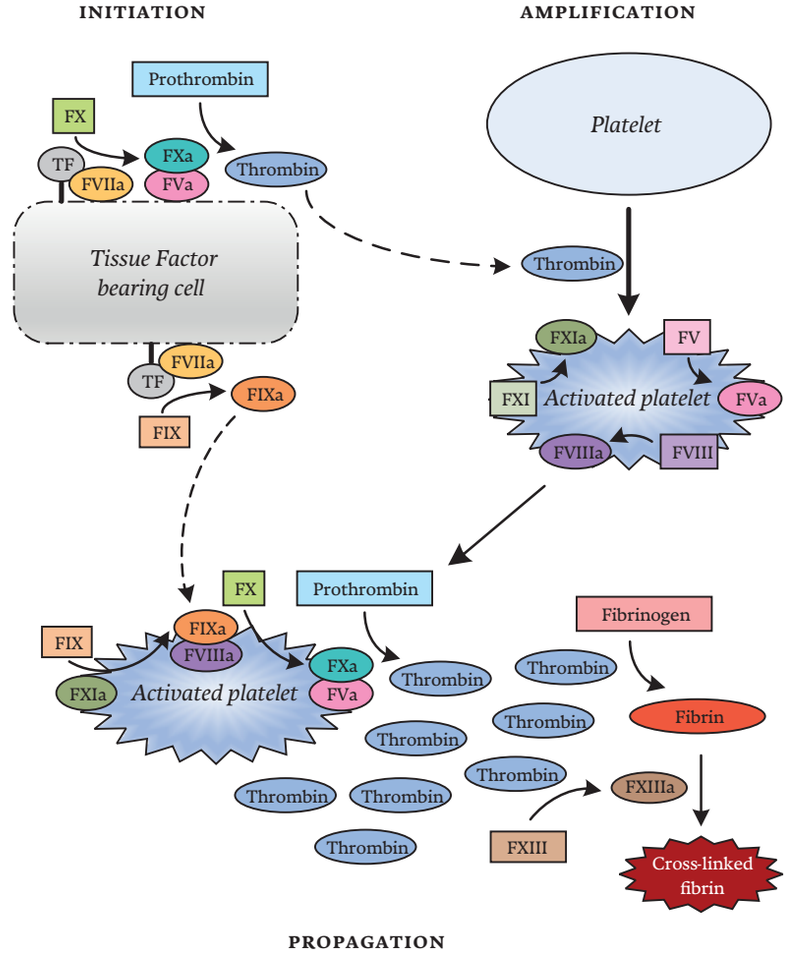
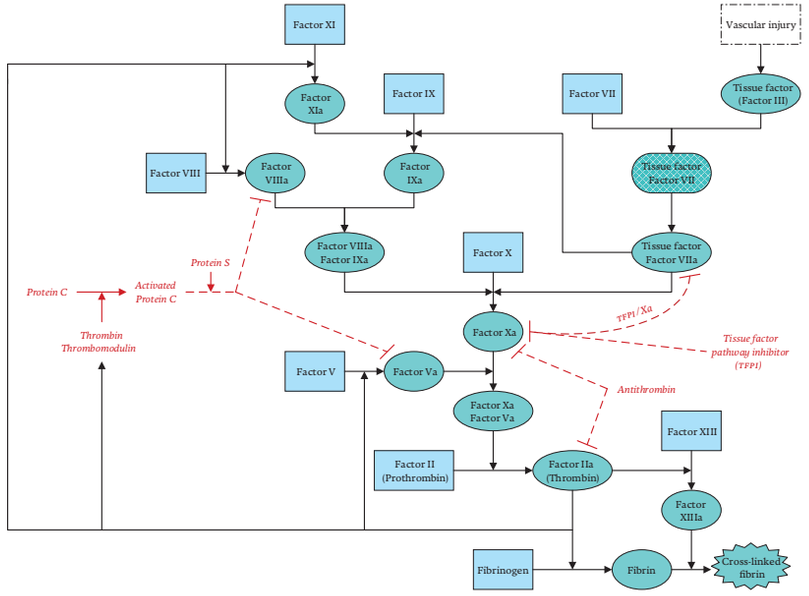


Figure 5 Schematic representation of the main activation and inhibition links between coagulation factors in the cell-based model of coagulation. When compared with the cascade model shown in Figure 3, initiation of coagulation in the cell-based model occurs via the extrinsic pathway only. By contrast, the intrinsic pathway mediates propagation of coagulation rather than serving as distinct activation pathway. Furthermore, additional activation routes are introduced in the cell-based model. For instance, extrinsic tenase is able to promote the activation of factor IX which connects the extrinsic pathway with the intrinsic pathway. In addition, thrombin enhances several upstream reactions, including activation of intrinsic factors XI and VIII, by positive feedback loops. Besides its procoagulant effects, thrombin is able to downregulate its own generation. Thrombin initiates an anticoagulant pathway by binding to thrombomodulin. Upon binding, thrombin enhances activation of protein C. Activated protein C, in conjunction with its cofactor protein S, inactivates factor Va and VIIIa. Other regulation mechanisms include tissue factor pathway (TFPI) and antithrombin. TFPI inhibits factor Xa directly and inactivates extrinsic tenase in a factor Xa dependent manner while antithrombin neutralizes factor Xa and thrombin. Solid arrows indicate activation and dashed lines indicate inhibition.



to nearby platelets to promote their full activation and to activate factor XI, VIII and V. Activated platelets propagate coagulation by providing a negatively charged surface at which the intrinsic tenase and prothrombinase complexes are efficiently assembled. This sets the stage for a burst of thrombin generation. The resulting thrombin converts fibrinogen into fibrin monomers and activates factor XIII yielding a stable clot. In addition to its procoagulant effects, thrombin also triggers anticoagulant mechanisms to maintain blood fluidity. This occurs when thrombin escapes from the site of injury without being inactivated by antithrombin, and binds to thrombomodulin on intact endothelial cells. Upon binding, the ability of thrombin to activate protein C increases while its procoagulant ability attenuates. Activated protein C, in conjunction with its cofactor protein S, inactivates factor Va and VIIIa (Figure 5).[35–37]

The cell based model has been a major advance in understanding the essential coagulation mechanisms *in vivo*. Nevertheless, other aspects of coagulation have been identified including thrombin inhibitor heparin cofactor II[36] and the positive feedback of factor VII activation by factor Xa.[38] Although not known to be essential for hemostasis, aspects of coagulation beyond the cell-based model may be relevant for pathological conditions and/or pharmacological intervention. In fact, for instance, Virchow's triad of risk factors for the formation of an occlusive clot within a blood vessel (thrombosis) underpins this. The risk factors hypercoagulability, stasis, and endothelial injury are not fully reflected by the cellular component and coagulation factors in the cell-based model.[39]

Sugammadex effects on coagulation

As part of sugammadex' non-clinical program, the potential effect of sugammadex on standard laboratory coagulation tests was investigated. Spiking experiments with fresh human plasma showed that sugammadex induces small prolongations of APTT and PT(INR). No indication of an increased bleeding risk was found in non-clinical safety studies. The *in vitro* off-target effect on coagulation parameters was not further evaluated in clinical studies. This was identified as important missing data by the European Medicines Agency (EMA) during their assessment of the application for marketing authorization for sugammadex in 2008. The EMA requested the applicant (then Schering-Plough) to investigate if sugammadex exposure increases bleeding risk in surgical patients. An analysis of adverse events in surgical patients who participated in placebo-controlled studies showed that the incidence of surgery related bleedings was comparable in the sugammadex (n=649) and the placebo (n=130) group, 2.8% and 2.3%, respectively (no statistically significant difference), indicating no predisposition of patients to bleeding complications

by sugammadex. Furthermore, Schering-Plough committed to perform post-authorization additional *in vitro* and *in vivo* studies to investigate the effect of sugammadex on coagulation.[40] This *in vitro* research is described in this thesis. The *in vivo* part included the assessment of sugammadex effects on APTT and PT(INR) in 8 healthy subjects. In this study, sugammadex induced a short-lasting (≤ 30 minutes) increase of 17% and 22% in APTT and an increase of 11% and 22% in PT(INR) following administration of 4 and 16 mg/kg sugammadex, respectively.[41] Concurrent with the EMA review, the United States Food and Drug Administration (FDA) initiated a Priority Review for sugammadex considering the potential significant benefit over existing reversal options.[42] The Anesthetic and Life Support Drugs Advisory Committee of the FDA unanimously recommended approval of sugammadex, however, a detailed review of the hypersensitivity data of sugammadex was not available at the time of their assessment.[43] In 2008, the FDA requested in their Not Approvable Letter further characterization of hypersensitivity reactions and the *in vitro* effects of sugammadex on coagulation markers.[44] To address the latter deficiency, Schering-Plough provided the FDA with the *in vitro* and *in vivo* research conducted to fulfill the post-authorization commitment to the EMA and analyses of available bleeding event data. Following review of this package, the FDA requested additional clinical trials.[42] This prompted the conduct of 2 sugammadex-drug interaction studies which are both covered by this thesis. In addition, a trial was performed in surgical patients receiving thromboprophylaxis and undergoing hip or knee joint replacement or hip fracture surgery. Sugammadex increased APTT and PT by 5.5% and 3.0%, respectively, as compared with usual care (neostigmine or spontaneous recovery) at 10 minutes after administration of the trial medication. The coagulation markers were fully normalized within 60 minutes after administration. These limited and transient effects did not translate into an increased bleeding risk within 24 hours and 14 days after surgery or into more blood loss post-operatively.[45]

Outline of this thesis

This thesis is comprised of a variety of *in vitro, ex vivo* and *in vivo* (clinical) pharmacology studies to provide more insight into the off-target effect of sugammadex on coagulation, with the aim to overcome the bleeding safety concerns raised by regulatory authorities.

CHAPTER 2 describes the experimental *in vitro* work initiated to unravel the mode of action by which sugammadex interferes with the coagulation cascade. This entailed evaluation of coagulation reactions such as factor Xa generation, factor Xa activity and thrombin generation in human plasma. In addition, it was investigated whether the APTT and PT effects of sugammadex are contributable to ORG 25969 and/or ORG 48302. The second and final part of the *in vitro* experiments is covered by **CHAPTER 3**. The *in vitro* effects of sugammadex on APTT and/or PT(INR) were explored in plasma of patients on a vitamin K antagonist with elevated INR's, in plasma of healthy subjects spiked with either a low or high level of enoxaparin, fondaparinux, rivaroxaban, and dabigatran and in perioperatively collected patient plasmas. Furthermore, the potential counteraction of sugammadex-induced APTT and PT prolongations by rocuronium or vecuronium was investigated.

In **CHAPTER 4**, an *ex vivo* whole blood collagen-induced platelet aggregation method was evaluated in healthy males. The relationship between collagen concentration and platelet aggregation was investigated, and assay reproducibility and intra-subject variability were assessed. In addition, the method was benchmarked by evaluating the effect of aspirin treatment. The method as described in **CHAPTER 4** was used to design the clinical study requested by the regulatory authorities to evaluate the effect of sugammadex co-administered with aspirin on platelet aggregation, APTT, cutaneous bleeding time and PT(INR), as presented in **CHAPTER 5**. This interaction study was performed in healthy males using a randomized, double-blind, placebo-controlled, 4-period cross-over study design.

CHAPTER 6 describes the results of an FDA-requested 2-part, randomized, double-blind, placebo-controlled, 4-period cross-over study in healthy males, evaluating the potential interaction effect between sugammadex and enoxaparin or unfractionated heparin (UFH) on anticoagulant activity. Anti-Xa activity and APTT were selected as primary endpoints parameters for enoxaparin and UFH, respectively, and as secondary endpoints *vice versa*. The exploratory endpoints included PT(INR) and pharmacokinetic/pharmacodynamic correlations.

Finally, **CHAPTER 7** combines the findings from the preceding chapters and places these in a broader perspective.

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