EVALUATING THE EFFECTS OF SUGAMMADEX ON COAGULATION IN HUMANS

REVERSED TRANSLATIONAL RESEARCH TO UNRAVEL OFF-TARGET PHARMACOLOGY

Annelieke C. Kruithof

EVALUATING THE EFFECTS OF SUGAMMADEX ON COAGULATION IN HUMANS

REVERSED TRANSLATIONAL RESEARCH TO UNRAVEL OFF-TARGET PHARMACOLOGY

Evaluating the effects of sugammadex on coagulation in humans

Reversed translational research to unravel off-target pharmacology

PROEFSCHRIFT
ter verkrijging van
de graad van doctor aan de Universiteit Leiden,
op gezag van rector magnificus prof.dr.ir. H. Bijl,
volgens besluit van het college voor promoties
te verdedigen op woensdag 15 december 2021
klokke 15:00 uur

DOOR
Annelieke Charissa Kruithof
geboren te Steenwijk
in 1985

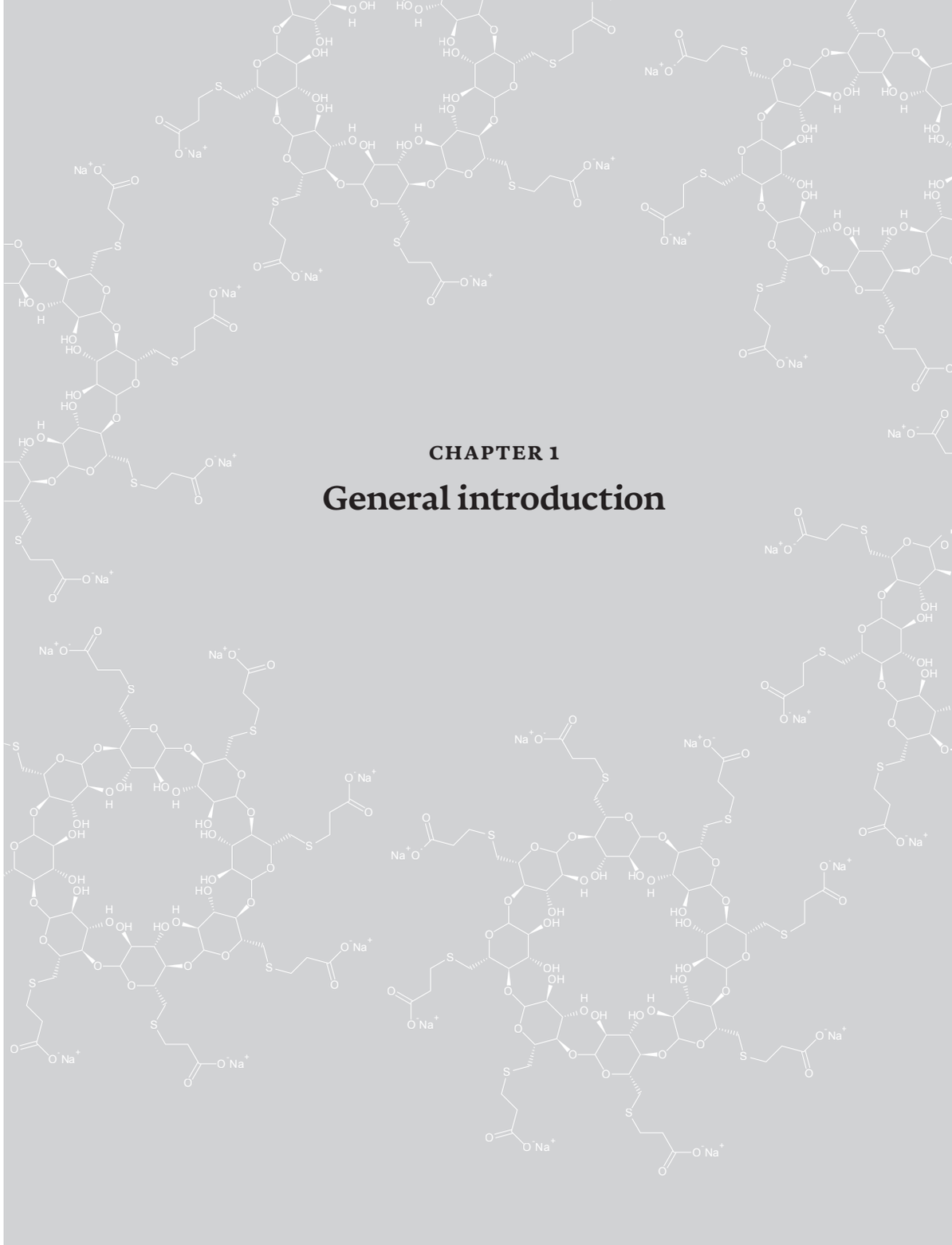
PROMOTORES
Prof. dr. J. Burggraaf
Prof. dr. C. Kluft (*Universiteit van Zuid-Denemarken*)

COPROMOTOR
Dr. M. Moerland

PROMOTIECOMMISSIE
Prof. dr. H.C.J. Eikenboom
Prof. dr. S.C. Cannegieter
Prof. dr. A. Dahan
Prof. dr. H. ten Cate (*Maastricht Universitair Medisch Centrum, Maastricht*)
Prof. dr. S. Middeldorp (*Radboud Universitair Medisch Centrum, Nijmegen*)

DESIGN
Caroline de Lint, Voorburg (caro@delint.nl)

The publication of this thesis was financially supported by the foundation Centre for Human Drug Research (CHDR) in Leiden, the Netherlands.



CHAPTER 1

General introduction

Neuromuscular blockade

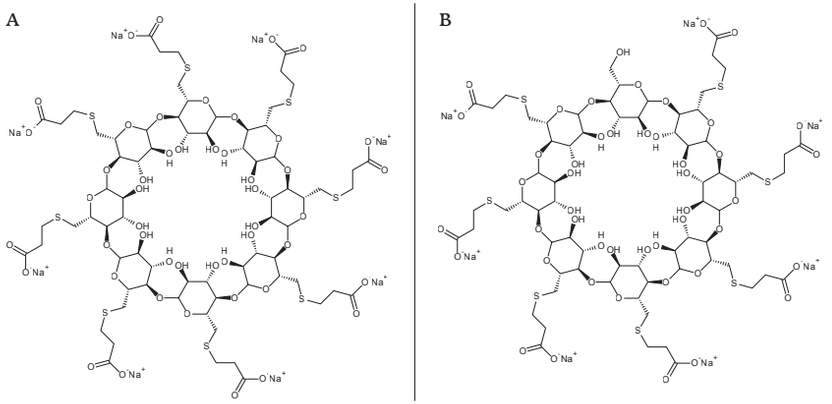
Neuromuscular blocking agents (NMBAs) are used in general anesthesia to facilitate tracheal intubation and achieve skeletal muscle relaxation during surgery.[1] The first NMBAs emerged mid-20th century and originate from curare, an extract of various plants used as paralyzing poison on arrow tips by 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.

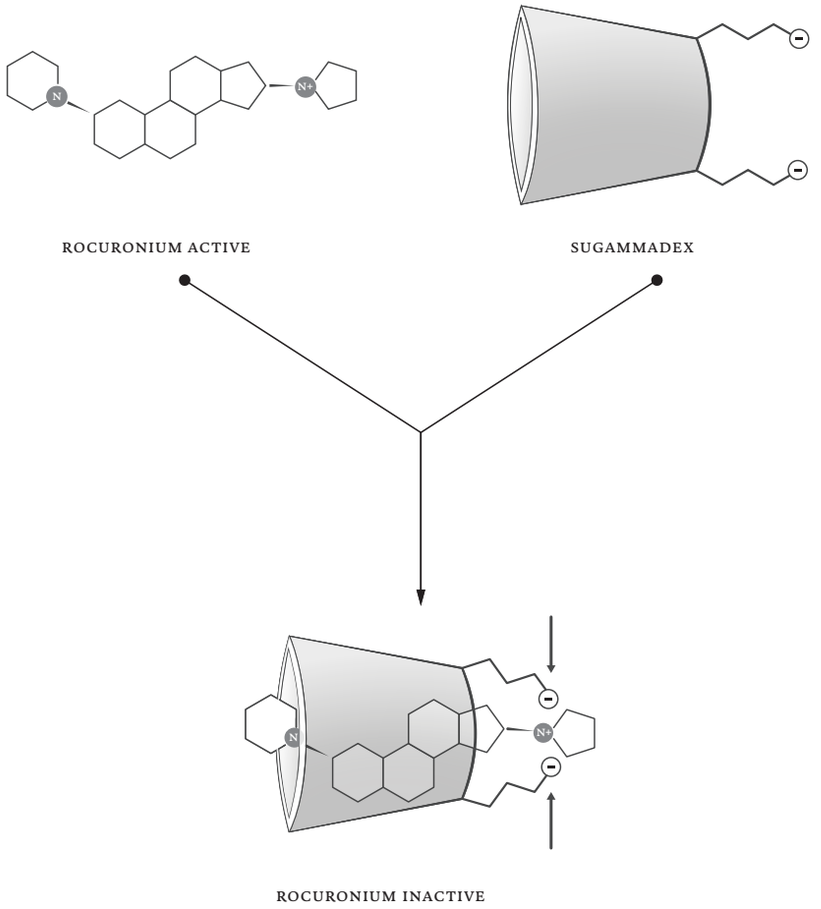


Sugammadex has a very high affinity for the steroidal NMBAs rocuronium and vecuronium, with a 2.5-fold difference in favor of the former,[13] and a moderate affinity for pancuronium (ORG NA97), but lacks affinity for the nonsteroidal NMBAs.[8] Sugammadex forms complexes with steroidal NMBAs with a 1:1 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.



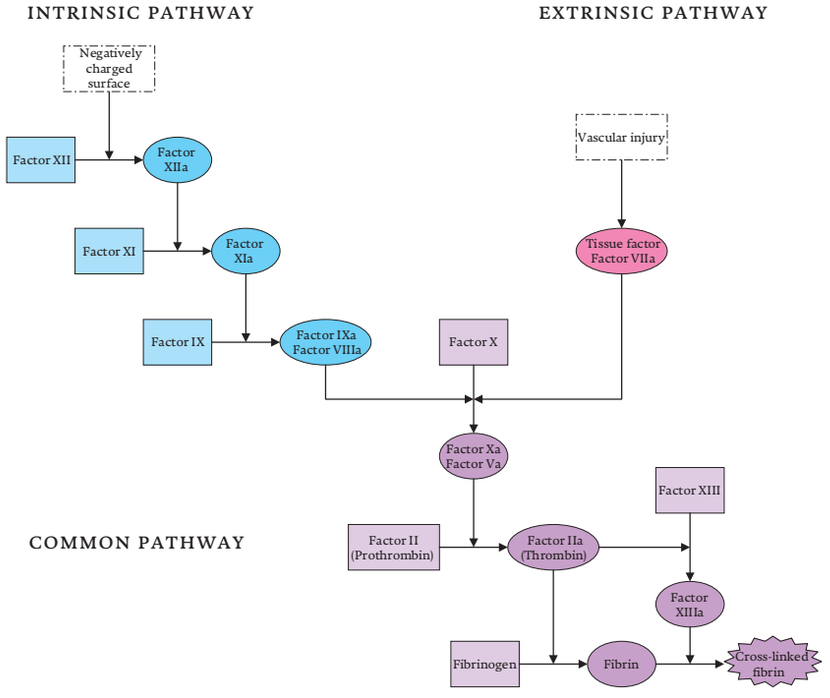
Before sugammadex became available for clinical use, its registration dossier was subject to regulatory review and approval. Potential safety concerns raised by regulatory agencies included the effect of sugammadex on coagulation and the clinical relevance thereof. This is described in more detail following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

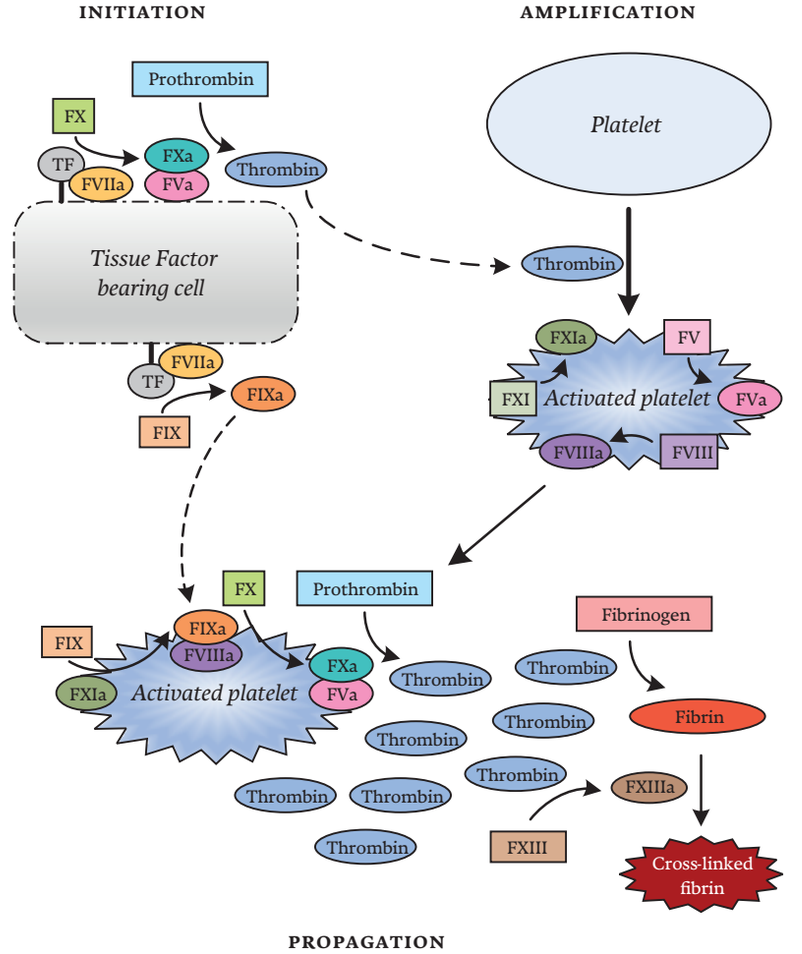
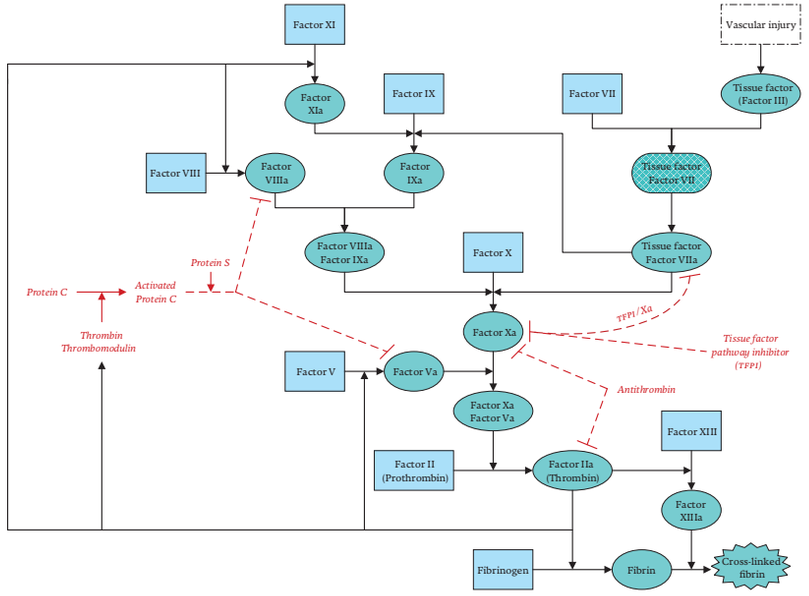


Figure 5 Schematic representation of the main activation and inhibition links between coagulation factors in the cell-based model of coagulation. When compared with the cascade model shown in Figure 3, initiation of coagulation in the cell-based model occurs via the extrinsic pathway only. By contrast, 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.

REFERENCES

1 W.T. Nicholson, J. Sprung, and C.J. Jankowski, *Sugammadex: a novel agent for the reversal of neuromuscular blockade.* Pharmacotherapy, 2007. 27(8): p. 1181-1188.

2 W.C. Bowman, *Neuromuscular block.* Br J Pharmacol, 2006. 147 Suppl 1: p. S277-286.

3 T. Raghavendra, *Neuromuscular blocking drugs: discovery and development.* J R Soc Med, 2002. 95(7): p. 363-367.

4 J.M. Hunter, *New neuromuscular blocking drugs.* N Engl J Med, 1995. 332(25): p. 1691-1699.

5 O. Lockridge, *Genetic variants of human serum cholinesterase influence metabolism of the muscle relaxant succinylcholine.* Pharmacol Ther, 1990. 47(1): p. 35-60.

6 E.W. Moore and J.M. Hunter, *The new neuromuscular blocking agents: do they offer any advantages?* Br J Anaesth, 2001. 87(6): p. 912-925.

7 L.H.D.J. Booij, H.D. de Boer, and J. van Egmond, *Reversal agents for nondepolarizing neuromuscular blockade: reasons for and development of a new concept.* Seminars in Anesthesia, Perioperative Medicine and Pain, 2002. 21(2): p. 92-98.

8 A. Srivastava and J.M. Hunter, *Reversal of neuromuscular block.* Br J Anaesth, 2009. 103(1): p. 115-129.

9 J.M. Adam, D.J. Bennett, A. Bom, J.K. Clark, *et al.*, *Cyclodextrin-derived host molecules as reversal agents for the neuromuscular blocker rocuronium bromide: synthesis and structure-activity relationships.* J Med Chem, 2002. 45(9): p. 1806-1816.

10 S.J. Brull and G.S. Murphy, *Residual neuromuscular block: lessons unlearned. Part II: methods to reduce the risk of residual weakness.* Anesth Analg, 2010. 111(1): p. 129-140.

11 B. Plaud, B. Debaene, F. Donati, and J. Marty, *Residual paralysis after emergence from anesthesia.* Anesthesiology, 2010. 112(4): p. 1013-1022.

12 M. Naguib, *Sugammadex: another milestone in clinical neuromuscular pharmacology.* Anesth Analg, 2007. 104(3): p. 575-581.

13 A. Bom, F. Hope, S. Rutherford, and K. Thomson, *Preclinical pharmacology of sugammadex.* J Crit Care, 2009. 24(1): p. 29-35.

14 L.H. Booij, *Cyclodextrins and the emergence of sugammadex.* Anaesthesia, 2009. 64 Suppl 1: p. 31-37.

15 Merck Sharp & Dohme Corp. *Label - Prescribing Information Bridion (sugammadex) Injection, for intravenous use*. 2015; Available from: https://www.accessdata.fda.gov/drugsatfda_docs/label/2015/022225lbl.pdf.

16 J.S. Jahr, J.E. Miller, J. Hiruma, K. Emaus, *et al.*, *Sugammadex: a scientific review including safety and efficacy, update on regulatory issues, and clinical use in Europe.* Am J Ther, 2015. 22(4): p. 288-297.

17 A. Abrishami, J. Ho, J. Wong, L. Yin, *et al.*, *Cochrane corner: sugammadex, a selective reversal medication for preventing postoperative residual neuromuscular blockade.* Anesth Analg, 2010. 110(4): p. 1239.

18 M. Carron, F. Zarantonello, P. Tellaroli, and C. Ori, *Efficacy and safety of sugammadex compared to neostigmine for reversal of neuromuscular blockade: a meta-analysis of randomized controlled trials.* J Clin Anesth, 2016. 35: p. 1-12.

19 W.J. Herring, T. Woo, C.A. Assaid, R.J. Lupinacci, *et al.*, *Sugammadex efficacy for reversal of rocuronium- and vecuronium-induced neuromuscular blockade: a pooled analysis of 26 studies.* J Clin Anesth, 2017. 41: p. 84-91.

20 S.J. Schaller and H. Lewald, *Clinical pharmacology and efficacy of sugammadex in the reversal of neuromuscular blockade.* Expert Opin Drug Metab Toxicol, 2016. 12(9): p. 1097-1108.

21 M. Welliver, *New drug sugammadex: a selective relaxant binding agent.* AANA J, 2006. 74(5): p. 357-363.

22 P. Duvaldestin, K. Kuizenga, V. Saldien, C. Claudius, *et al.*, *A randomized, dose-response study of sugammadex given for the reversal of deep rocuronium- or vecuronium-induced neuromuscular blockade under sevoflurane anesthesia.* Anesth Analg, 2010. 110(1): p. 74-82.

23 F.K. Puhringer, M. Gordon, I. Demeyer, H.J. Sparr, *et al.*, *Sugammadex rapidly reverses moderate rocuronium- or vecuronium-induced neuromuscular block during sevoflurane anaesthesia: a dose-response relationship.* Br J Anaesth, 2010. 105(5): p. 610-619.

24 K. Suy, K. Morias, G. Cammu, P. Hans, *et al.*, *Effective reversal of moderate rocuronium- or vecuronium-induced neuromuscular block with sugammadex, a selective relaxant binding agent.* Anesthesiology, 2007. 106(2): p. 283-288.

25 Merck Sharp & Dohme Limited. *Bridion Annex I: Summary of Product Characteristics*. 2018; Available from: https://www.ema.europa.eu/documents/product-information/bridion-epar-product-information_en.pdf.

26 P. Peeters, P. Passier, J. Smeets, A. Zwiers, *et al.*, *Sugammadex is cleared rapidly and primarily unchanged via renal excretion.* Biopharm Drug Dispos, 2011. 32(3): p. 159-167.

27 F. Gijsenbergh, S. Ramael, N. Houwing, and T. van Iersel, *First human exposure of Org 25969, a novel agent to reverse the action of rocuronium bromide.* Anesthesiology, 2005. 103(4): p. 695-703.

28 G.M. Keating, *Sugammadex: a review of neuromuscular blockade reversal.* Drugs, 2016. 76(10): p. 1041-1052.

29 K.J. Clemetson, *Platelets and primary haemostasis.* Thromb Res, 2012. 129(3): p. 220-224.

30 G. Lippi, E.J. Favaloro, M. Franchini, and G.C. Guidi, *Milestones and perspectives in coagulation and hemostasis.* Semin Thromb Hemost, 2009. 35(01): p. 009-022.

31 D.S. Minors, *Haemostasis, blood platelets and coagulation.* Anaesthesia & Intensive Care Medicine, 2007. 8(5): p. 214-216.

32 A.L. Bloom, *Physiology of blood coagulation.* Haemostasis, 1990. 20 Suppl 1: p. 14-29.

33 H.M. Spronk, J.W. Govers-Riemslag, and H. ten Cate, *The blood coagulation system as a molecular machine.* Bioessays, 2003. 25(12): p. 1220-1228.

34 A.H. Kamal, A. Tefferi, and R.K. Pruthi, *How to interpret and pursue an abnormal prothrombin time, activated partial thromboplastin time, and bleeding time in adults.* Mayo Clin Proc, 2007. 82(7): p. 864-873.

35 M. Hoffman and D.M. Monroe III, *A cell-based model of hemostasis.* Thromb Haemost, 2001. 85(6): p. 958-965.

36 H.H. Versteeg, J.W.M. Heemskerk, M. Levi, and P.H. Reitsma, *New fundamentals in hemostasis.* Physiol Rev, 2013. 93(1): p. 327-358.

37 J.S. O'Donnell, J.M. O'Sullivan, and R.J.S. Preston, *Advances in understanding the molecular mechanisms that maintain normal haemostasis.* Br J Haematol, 2019. 186(1): p. 24-36.

38 A.M. Shibeko, E.S. Lobanova, M.A. Panteleev, and F.I. Ataullakhanov, *Blood flow controls coagulation onset via the positive feedback of factor VII activation by factor Xa.* BMC Syst Biol, 2010. 4(5): p. 1-12.

39 K.G. Mann, K. Brummel-Ziedins, T. Orfeo, and S. Butenas, *Models of blood coagulation.* Blood Cells Mol Dis, 2006. 36(2): p. 108-117.

40 European Medicines Agency. *Assessment Report for Bridion*. 2008; Available from: www.ema.europa.eu/docs/en_GB/document_library/EPAR_-_Public_assessment_report/human/000885/WC500052309.pdf.

41 P.J. de Kam, P. Grobara, M. Prohn, F. Höppener, *et al.*, *Effects of sugammadex on activated partial thromboplastin time and prothrombin time in healthy subjects.* Int J Clin Pharmacol Ther, 2014. 52(3): p. 227-236.

42 Food and Drug Administration Center for Drug Evaluation and Research. *Application Number: 022225Orig1s000, Medical Review(s).* 2015; Available from: https://www.accessdata.fda.gov/drugsatfda_docs/nda/2015/022225Orig1s000MedR.pdf.

43 Food and Drug Administration Center for Drug Evaluation and Research. *Background Materials; Meeting of the Anesthetic and Analgesic Drug Products Advisory Committee (AADPAC).* 2015; Available from: https://www.fda.gov/AdvisoryCommittees/CommitteesMeetingMaterials/Drugs/AnestheticAndAnalgesicDrugProductsAdvisoryCommittee/ucm470714.htm.

44 Food and Drug Administration Center for Drug Evaluation and Research. *Application Number: 022225 Orig1s000, Other Action Letters*. 2015; Available from: https://www.accessdata.fda.gov/drugsatfda_docs/nda/2015/022225Orig1s000OtherActionLtr.pdf.

45 N. Rahe-Meyer, H. Fennema, S. Schulman, W. Klimscha, *et al.*, *Effect of reversal of neuromuscular blockade with sugammadex versus usual care on bleeding risk in a randomized study of surgical patients.* Anesthesiology, 2014. 121(5): p. 969-977.

CHAPTER 2

The mode of action of sugammadex on coagulation

International Journal of Clinical Pharmacology and Therapeutics, 2021. 59(2): p. 89-98, doi: 10.5414/CP203858

Annelieke C. Kruithof, Cornelis Kluft, Pieter-Jan de Kam, Geertruida H. Laterveer, Matthijs Moerland, and Jacobus Burggraaf

Abstract

OBJECTIVE To explore the mode of action (MoA) by which sugammadex interferes with coagulation.

MATERIALS AND METHODS The effect of sugammadex on various steps in the coagulation cascade including thrombin generation, factor Xa activity and factor Xa generation was explored in human plasma.

RESULTS Sugammadex did not affect a conventional thrombin generation test (TGT) while it prolongs activated partial thromboplastin time (APTT) and prothrombin time (PT). However, a customized TGT with PT reagent revealed sugammadex effects. In addition, sugammadex prolonged a one-step prothrombinase induced clotting time (PiCT) using human factor Xa. Furthermore, sugammadex interfered with factor Xa generation induced by an intrinsic and not by an extrinsic activator, nor by Russell's Viper Venom factor X (RVV-X).

CONCLUSION Adapted, rather than standard experiments, show that sugammadex is likely to decrease factor Xa activity in the common pathway and activation of factor X specifically in the intrinsic pathway.

Introduction

Sugammadex (Bridion®, Merck Sharp & Dohme Corp., Oss, The Netherlands) is a modified γ-cyclodextrin that encapsulates specifically the steroidal neuromuscular blocking agents (NMBAs) rocuronium and vecuronium and thereby reverses the neuromuscular blockade induced by these compounds in the post-operative setting. The recommended dose is 2 and 4 mg/kg sugammadex for routine reversal of moderate and deep block, respectively; a dose of 16 mg/kg sugammadex is effective for reversal 3 minutes after an intubating dose of 1.2 mg/kg rocuronium.

A prior study in 8 healthy volunteers suggested that, in the absence of a NMBA, sugammadex caused dose-dependent short duration (≤ 30 minutes) increases in activated partial thromboplastin time (APTT) and prothrombin time (PT). In this study, sugammadex demonstrated a prolongation of 17% and 22% in APTT and a prolongation of 11% and 22% in PT after administration of 4 and 16 mg/kg, respectively, compared with placebo.[1] Limited and transient (< 1 hour) increases in APTT and PT were also observed in a prospective study in 1,198 surgical patients at risk for bleeding complications following the use of sugammadex 4 mg/kg *versus* usual care (neostigmine or spontaneous recovery). The results of this study demonstrated that the use of sugammadex is not associated with an increased risk of bleeding complications.[2] Nonetheless, there is a need to determine the Mode of Action (MoA) by which sugammadex prolongs APTT and PT. The drug substance sugammadex contains mainly the modified γ-cyclodextrin ORG 25969 and some traces of its related γ-cyclodextrin ORG 48302. We scrutinized which of these components is primarily responsible for the observed APTT and PT increases. Subsequently, we performed *in vitro* coagulation experiments to elucidate the MoA by which sugammadex interferes with the coagulation cascade. As briefly described previously, we were eventually able to demonstrate the effects on APTT and PT may be explained by a sugammadex-induced decrease in factor Xa activity in the common pathway and activation of factor X specifically in the intrinsic pathway.[1] In order to unravel this MoA, we employed customized clotting assays as the results obtained with conventional assays were partly ambiguous. The full experimental stepwise approach has not previously been disclosed and is described here in detail.

Materials and methods

Investigations were performed at the laboratories of Good Biomarker Sciences (GBS) in close collaboration with the Centre for Human Drug Research (CHDR; both Leiden, The Netherlands). Blood from 12 consenting, apparently healthy volunteers was drawn into citrated (0.105 M) tubes and centrifuged twice for 20 minutes at 2000 × g at 4 °C. After pooling plasma of 6 subjects, aliquots were stored at −60 °C and thawed only once prior to use. Plasma was spiked with sugammadex (ORG 25969) or its related γ-cyclodextrin ORG 48302, which are present at > 93% and < 7% in the drug substance sugammadex, respectively,[3] both kindly provided by Merck Sharp & Dohme Corp. (formerly known as Schering-Plough), Oss, The Netherlands. Normal and spiked plasma were used to perform a variety of coagulation assays. Clotting times were recorded with an automated clotting analyzer AMAX Destiny Plus (Kordia Life Sciences, Leiden, The Netherlands), unless specified otherwise. All measurements were performed in duplicate.

APTT AND PT

APTT was assessed using an APTT kit from bioMérieux, Marcy l'Etoille, France. In short, 25 µL plasma was pre-incubated with 25 µL automated APTT reagent containing rabbit brain phospholipids and microsilica activator. Clotting was initiated by adding 25 µL 0.025 M $CaCl_2$ solution. PT was measured by mixing 25 µL plasma with 50 µL Simplastin HTF reagent containing human tissue factor (TF) thromboplastin derived from cultured human cells and calcium ions (bioMérieux, Marcy l'Etoille, France) to initiate extrinsic clotting.

In addition, adapted APTT and PT assays were employed. APTT supplemented with Russell's Viper Venom factor V (RVV-V) was performed by adding RVV-V activator (Pentapharm, Kordia Life Sciences, Leiden, The Netherlands) to plasma immediate before APTT testing. APTT was also measured in factor V deficient plasma (Haematologic Technologies Inc., Bio-Connect B.V., Huissen, The Netherlands). Furthermore, PT reagent was supplemented with activated factor VII. Therefore, an ampoule of 2.4 mg NovoSeven® (120 KIE, Novo Nordisk A/S, Bagsværd, Denmark) was dissolved according to manufacturer's instructions and diluted with NaCl 0.9% solution containing 0.1% BSA to 124 nM.

Controls used for APTT and PT were Accuclot Control I and II and Verify 1 and 2 (bioMérieux, Marcy l'Etoille, France).

ECARIN CLOTTING TIME

Ecarin Clotting Time (ECT; with HEPES buffer and Accuclot Controls) was performed by incubating 50 µL plasma with an Ecarin concentration (50 EU/vial, Pentapharm, Kordia Life Sciences, Leiden, The Netherlands) resulting in clotting time of ≈20 seconds.

THROMBIN GENERATION

First, a thrombin generation test (TGT) was performed using a fluorogenic assay. This was carried out with the Technothrombin® TGA kit (Technoclone, Kordia Life Sciences, Leiden, The Netherlands) and corresponding TGA RCH reagent with high phospholipid concentration and 0.5 pM TF. Fluorescence generated by thrombin cleavage of the fluorogenic thrombin substrate was recorded every 30 seconds with the Fluostar Galaxy (BMG, Offenburg, Germany) and expressed in arbitrary units (AU). The TGT assay was performed in normal and defibrinated plasma. Defibrination was achieved by addition of 6 µL reptilase (Pentapharm, Kordia Life Sciences, Leiden, The Netherlands) to 150 µL plasma followed by incubation of the mixture at 37 °C. After 10 minutes, it was kept on melting ice for an additional 10 minutes, after which the fibrin clot was removed.

The TGT was adapted to mimic PT by replacing RCH with 1:4-diluted Simplastin HTF reagent used for PT and a shortened recording interval of 20 seconds. To further mimic the PT test, a TGT with a high level TF was performed by mixing 30 µL plasma with 30 µL PT reagent Innovin (Dade Behring, Marburg, Germany), using 30 µL fluorogenic TGA substrate and a recording interval of 15 seconds.

Besides the fluorogenic TGT assays, a chromogenic TGT assay (Pefakit inTDT, Pentapharm, Basel, Switzerland) was carried out after replacing its starting reagent with 50 µL human factor Xa (1%, Bio-Connect B.V., Huissen, The Netherlands) and 10 µL rabbit brain cephalin (500 µM, Pentapharm, Kordia Life Sciences, Leiden, The Netherlands) as activator. Thrombin formation was monitored by the increase of absorbance at 405 nm which accompanies the release of the chromophore from the chromogenic thrombin substrate by thrombin. Absorbance was recorded with a microplate reader (Tecan Benelux BVBA, Giessen, The Netherlands) every 7 seconds. The first derivative of the resulting curve was calculated, representing the velocity of the conversion of the chromogenic substrate by thrombin.

FACTOR XA ACTIVITY

A human one-step prothrombinase induced clotting time (PiCT) was designed using separate reagents aiming at comparable clotting times as with a commercial bovine two-step PiCT test (Pefakit PiCT kit, Pentapharm, Basel, Switzerland) modified into a one-step variant. A one-step PiCT is performed by freshly premixing both reagents and then starting clotting by adding this mix to plasma.[4] The details of composition of the bovine PiCT reagents were undisclosed, but the source of phospholipids (rabbit brain cephalin) and RVV-V was anticipated to be from the same manufacturer and subsequently purchased separately (Pentapharm, Kordia Life Sciences, Leiden, The Netherlands). PiCT reagent was prepared for 10 measurements by mixing 160 µL 500 µM rabbit brain cephalin, 16 µL 1000 U/mL RVV-V, 800 µL 0.025 M $CaCl_2$, 622 µL HEPES buffer and 2 µL purified human factor Xa (100 µg/mL, Hyphen Biomed, Neuville-sur-Oise, France). This reagent was added to 80 µL plasma.

FACTOR XA GENERATION

Factor Xa generation was induced by RVV factor X (RVV-X) and via the extrinsic and intrinsic coagulation routes in a chromogenic factor Xa generation assay. In a microplate, a mixture of 5 µL plasma, 110 µL PEG/Ca^{2+} buffer (pH 7.5), 5 µL ORG 25969 solution or buffer, and 50 µL chromogenic substrate for factor Xa Spectrozyme FXa (1.6 mM in PEG/Ca^{2+} buffer, American Diagnostica INC, Stamford, Connecticut United States of America (USA)) was incubated at 25 °C. Factor Xa generation was initiated by addition of 25 µL PT reagent Neoplastin Plus (Diagnostica Stago, Asnières sur Seine, France) for extrinsic factor Xa generation. The RVV-X mediated and intrinsic factor Xa generation variants were performed by substituting the PT reagent with RVV-X (Enzyme Research Laboratories Ltd, Swansea, United Kingdom) and automated APTT reagent, respectively, and increasing the plasma volume to 10 µL at expense of the buffer.

Factor Xa generation was monitored by the increase of absorbance at 405 nm which accompanies the release of the chromophore from the chromogenic factor Xa substrate by factor Xa. Absorbance was recorded on a microplate reader every 30 seconds for a period of 1 hour. The first derivative of the resulting curve was calculated, representing the velocity of the conversion of the chromogenic substrate by factor Xa.

All measurements were performed with an excess of recombinant hirudin (5 µL 400 U/mL, Pentapharm, Basel, Switzerland) to exclude participation of thrombin and thrombin-mediated factor V activation. This suppression was substantiated by performing the extrinsic variant with prothrombin and factor V deficient plasma (both from Haematologic Technologies Inc., Bio-Connect B.V., Huissen, The Netherlands) which yielded similar results to normal plasma. In addition, rivaroxaban (Tanabe Seiyaku Co., Ltd., Osaka, Japan) was used to establish that the observed peak is primarily factor Xa generation.

A direct effect of ORG 25969 on the chromogenic substrate was excluded as the activity of purified factor Xa in absence and presence of ORG 25969 was comparable.

Results

SCREENING CLOTTING ASSAYS

The effects of ORG 25969 and its related γ-cyclodextrin ORG 48302 on APTT and PT were assessed in pooled plasma of healthy volunteers (Figure 1). Both components of the drug substance sugammadex prolonged APTT and PT in a concentration-dependent manner with the strongest effect observed for ORG 25969. ORG 25969 increased APTT and PT with ≈10 and 2.5 seconds, respectively, at a concentration of 200 µg/mL, which is the anticipated mean peak plasma concentration following the highest therapeutic dose of sugammadex (16 mg/kg). At the concentration of ORG 48302 that would be present after administration of this sugammadex dose (± 14 µg/mL assuming sugammadex contains 7% ORG 48302) only minimal prolongations in APTT (0.3 seconds) and PT (0.2 seconds) appear to occur. As the clinical APTT and PT prolongations would be mainly driven by ORG 25969, this γ-cyclodextrin was selected for the subsequent assays.

THROMBIN ACTIVITY

Previous investigations revealed no effect of ORG 25969 on the conversion of fibrinogen into fibrin by thrombin using the thrombin time assay (unpublished data) suggesting no thrombin activity inhibition by ORG 25969. This was further substantiated by measuring the effect of ORG 25969 on the same conversion but then by meizothrombin using the ECT assay. ORG 25969 did not substantially affect this assay indicating no direct thrombin inhibitor-like effect of ORG 25969 (18.8 and 19.1 seconds for 0 and 500 µg/mL ORG 25969, respectively).

THROMBIN GENERATION

Next, it was investigated whether ORG 25969 affects thrombin generation using fluorogenic TGT assays. The initial fluorogenic TGT was performed using the Technoclone TGA with RCH reagent. Remarkably, no effect of ORG 25969 was observed in the fluorogenic TGT in both normal and defibrinated plasmas (data on file) while ORG 25969 did induce PT effects in normal plasma. This may be explained, amongst others, by differences in source and concentration of TF and/or phospholipids used to activate these assays; for instance the TF concentration in TGT reagent is much lower compared to PT (pM and nM range, respectively). Therefore, we aimed to resemble the conditions of the PT by replacing the RCH reagent with diluted Simplastin HTF reagent used for PT. However, ORG 25969 added up to 600 µg/mL to normal plasma yielded similar results (data on file). The next step to mimic the PT conditions was to minimize the difference in duration between TGT (minutes) and PT (seconds) by designing an experimental TGT assay with the shortest possible recording interval (15 seconds) and undiluted PT reagent (Innovin). In this assay, thrombin mediated fluorescence intensity increased less rapidly with ORG 25969 (400 µg/mL) than without it (Figure 2).

Apparently, the effects of ORG 25969 remain when the assay read-out is fluorescence intensity generated by thrombin cleavage of a synthetic thrombin substrate instead of assessment of clot formation. Taking this into account, together with the fact that APTT and PT were both affected by ORG 25969, ORG 25969 is likely to interfere with the common pathway of the coagulation cascade and this was subsequently explored.

FACTOR XA ACTIVITY

The potential factor Xa-inhibiting characteristics of ORG 25969 were explored with one-step PiCT tests.[4] Species difference in sensitivity to factor Xa inhibition has been previously described for instance for direct factor Xa inhibitors in humans, rabbits, dogs and rats.[5–8] Hence, we performed a PiCT with human factor Xa and observed a clear concentration-dependent inhibitory effect of ORG 25969 (Figure 3).

Since ORG 25969 appeared to interfere with the prothrombin-to-thrombin conversion by inhibiting factor Xa activity, the possible involvement of factor V in Org 25969's inhibitory effect on coagulation was investigated next. In factor V deficient plasma, addition of 200 µg/mL ORG 25969 prolonged APTT from 138.8 (± 2.1) to 170.7 (± 1.0) seconds which is in line with the corresponding relative APTT prolongation observed in normal plasma (Figure 1). In plasma with increased factor V activation due to addition of RVV-V, ORG 25969 still prolonged APTT compared to control plasma, although the difference seemed to become smaller with increasing factor V activation. Addition of 2% (v/v) RVV-V 1,000 U/mL decreased APTT from 48.2 (± 1.5) to 32.5 (± 0.5) seconds compared to 40.1 (± 0.2) to 29.9 (± 0.3) seconds, in the absence and presence of 200 µg/mL ORG 25969, respectively. Doubling RVV-V yielded a further APTT decrease of 10% for both conditions with 27.0 (± 0.1) and 29.1 (± 0.0) seconds, respectively. The potential modulating role of factor Va was not further investigated.

The effect of ORG 25969 on factor Xa activity was further scrutinized by testing whether eliminating the positive feedback of factor VII activation by factor Xa could suppress ORG 25969 induced PT prolongation. Factor VII activation, including factor Xa-mediated activation, can be reduced by adding factor VIIa. Enrichment of control plasma with recombinant factor VIIa (NovoSeven®) up to 3.1, 6.2 and 12.4 nM shortened PT from 13.5 to 9.0–9.2 seconds. The PT shortening was less pronounced in the presence of 200 µg/mL ORG 25969 (from 14.0 to 11.8–11.9 seconds following enhancement of factor VIIa) suggesting that the positive feedback of factor VII activation by factor Xa is not key to ORG 25969 effects on factor Xa activity.

FACTOR XA GENERATION

Factor Xa generation can be induced via extrinsic and intrinsic coagulation routes or by metalloprotease RVV-X. The effect of ORG 25969 on all these activation routes was investigated using chromogenic factor Xa generation assays.

First, an experimental extrinsic factor Xa generation assay was set-up with PT reagent. In this assay, ORG 25969 did not inhibit the formation of factor Xa (data on file). Second, ORG 25969 was tested in the intrinsic route of factor Xa generation (using APTT reagent) and was found able to inhibit factor Xa generation in a concentration-dependent manner (Figure 4). Third, no ORG 25969 induced inhibition was observed in RVV-X induced factor Xa generation (data on file). Hence, ORG 25969 specifically inhibits factor Xa generation via the intrinsic activation coagulation route.

RECONCILIATION EXPERIMENT

The above described experiments generate the hypothesis that ORG 25969 may inhibit factor Xa activity in the common pathway and activation of

factor X specifically in the intrinsic pathway. In consideration of this potential MoA, we designed an experimental chromogenic TGT with human factor Xa. A chromogenic variant was preferred over a fluorogenic TGT as the shortest possible recording interval of a spectrophotometer is 7 seconds compared to 15 seconds with a fluorimeter. We modified a commercial TGT by using human factor Xa as starting agent and cephalin as phospholipid source. In this assay, ORG 25969 caused a concentration-dependent prolongation of lag phase and a minor decrease in peak height with no appreciable effect on rate of thrombin formation (Figure 5).

Discussion

Previously, sugammadex was found to be associated with a limited, concentration-dependent prolongation of APTT and PT in a clinical study in healthy volunteers. These increases were shown to be only transient (< 30 minutes) in parallel with the rapid decrease of sugammadex plasma level.[1] Because sugammadex is used in the surgical setting where bleeding is a concern, these findings prompted a dedicated prospective study of 4 mg/kg sugammadex in surgical patients at risk for bleeding complications. This study demonstrated that sugammadex is not associated with an increased risk of bleeding complications.[2] To further evaluate the potential impact of the effects of sugammadex on coagulation, we investigated a variety of coagulation experiments to explore the possible underlying MoA. We selected human plasma as matrix to mimic the clinical situation as much as possible. These experiments, briefly reported previously,[1] are described here in detail.

The drug substance sugammadex contains mainly the modified γ-cyclodextrin ORG 25969 and some traces of its related γ-cyclodextrin ORG 48302.[3] The present findings showed that the key component of sugammadex is noticeably more potent than ORG 48302 in prolonging clotting readout measures APTT and PT. The components only differ in degree of substitution; ORG 25969 and ORG 48302 have 8 and 7 carboxyl thioether groups, respectively, indicating that small differences in degree of substitution and related charge density may be relevant for the effect on coagulation. It has been previously reported that chemically related sulfated cyclodextrins prolong APTT in human[9–11] and animal[12,13] material, although it cannot be considered as class effect as for instance sulfobutyl-ether derivatives of β-cyclodextrin with a substitution degree of 1 or 4 have been shown not to increase APTT.[11] In view of the substantial difference in magnitude of effect in APTT and PT by ORG 25969 and ORG 48302, we selected only ORG 25969 for the mechanistic experiments.

Based on the polyanionic saccharide nature of ORG 25969,[14] a heparinoid-like anticoagulation effect was considered to be a plausible MoA. Heparinoids such as heparin and dermatan sulfate primarily exert their anticoagulant effect via thrombin inhibitors antithrombin and heparin cofactor II, respectively.[15] If, for instance, the carboxylic acid groups of the carboxyl thioether groups of ORG 25969 are spaced appropriately, this may provide a similar electron density template for interaction with antithrombin as the core pentasaccharide of heparin.[16] A similar type of interaction may serve as underlying mechanism in which case ORG 25969 would potentiate heparin cofactor II, for which it has been described that it can be potentiated by various polyanions such as sulfated compounds.[17–19] Nevertheless, these possible MoAs were excluded as we found similar ORG 25969 concentration *versus* APTT/PT effect profiles in normal pooled and antithrombin or heparin cofactor II depleted plasmas.[1] Apparently, ORG 25969 has a different mechanism by which it influences coagulation.

To generate a hypothesis on its possible MoA, the effect of ORG 25969 on the coagulation cascade was investigated upstream-wise, starting with the conversion of fibrinogen into fibrin by (meizo-)thrombin. No effect on (meizo-)thrombin activity was observed, so fibrin formation appeared to remain intact in presence of ORG 25969.

Next, thrombin generation was explored. Surprisingly, ORG 25969 was unable to induce a detectable effect in the TGT while this assay is initiated in a similar way as PT, although PT is more strongly activated due to an order of magnitude higher TF concentration resulting in a shorter assay time compared to TGT (seconds *versus* minutes, respectively). Replacing the TGT reagent with the same reagent as used for PT still yielded no noticeable ORG 25969 effect. To further mimic the PT, an experimental TGT was designed that lasted approximately 1 minute. In this setting, thrombin mediated fluorescence intensity increased less rapidly in the presence of ORG 25969. This is rather counterintuitive as one would expect an inhibitor to be more effective on reactions with a weaker activator than those with a stronger activator. To our knowledge, no similar TGT observation has been reported before. Further investigations explaining this observation should be directed at the effect of the reagent composition and include coagulation inhibitors with a known MoA such as a direct factor Xa inhibitor for comparison purposes.

As eventually an effect on TGT by ORG 25969 was observed, and taking into account the APTT and PT prolongations, the experimental focus shifted to the more upstream factor Xa activity. Considering the known species difference in sensitivity to factor Xa inhibition,[5–8] human factor Xa is preferably used to investigate the potential factor Xa inhibitory effect of ORG 25969. Therefore, a customized PiCT with human factor Xa was assembled. In this assay, clotting time increased with raising ORG 25969 concentration, indicating that factor Xa activity is hampered by ORG 25969.

As factor Va functions as a cofactor to factor Xa in the conversion of prothrombin to thrombin, the effect of ORG 25969 on factor V(a) was investigated next. Factor V appeared not to be a key determinant of the anticoagulant effect of ORG 25969. In contrast, factor Va seemed to be able to counteract the inhibition of factor Xa activity which may suggest that ORG 25969 is less able to interfere with factor Xa activity when factor Xa is complexed with factor Va compared to non-complexed factor Xa.

Factor Xa is also capable of activating factor VII, and this feedback activation may be targeted by ORG 25969 as well. However, ORG 25969 was still able to exert its inhibitory effect on PT with increasing factor VIIa concentrations suggesting that factor Xa activation of factor VII is not essential for ORG 25969 effects on factor Xa activity.

Subsequently, the more upstream factor Xa generation was explored. ORG 25969 was able to suppress intrinsic factor Xa generation in a concentration-dependent manner while the extrinsic and RVV-X induced factor Xa generation were unaffected.

From our findings, we postulated that ORG 25969 may inhibit factor Xa activity in the common pathway and activation of factor X specifically in the intrinsic pathway. This was further substantiated by an experimental TGT with human factor Xa as activator and cephalin showing a concentration-dependent delay of thrombin generation by ORG 25969. In addition, the maximum thrombin generation appeared slightly diminished and the rate unaffected.

Dirkmann *et al.* interpreted the anticoagulant effect of sugammadex likely to be an *in vitro* artifact. They added sugammadex to blood collected from healthy subjects and observed prolongation of APTT, PT, and clotting time in extrinsically and intrinsically activated thromboelastometric assays. Addition of sugammadex also reduced activities of factors VIII, IX, XI and XII. Several other assays were unaffected including the thrombin generation assay, clot firmness, clot lysis, fibrinogen concentration, and activities of other coagulation factors. The hypothesis emerged that the observed effects could be explained by a phospholipid-binding effect. This was explored by performing a phospholipid-sensitive APTT and diluted Russel viper venom time assay (DRVVT); the latter with low and high phospholipid concentration. Sugammadex prolonged all assays with a diminished effect in the DRVVT with a high phospholipid concentration, although the effect was not completely abolished by enhancement of phospholipids rendering insufficient information for a conclusive interpretation.[20] We previously reported no/limited effects by ORG 25969 on clotting time upon dilution up to 1,000-fold of the PT reagent[1] suggesting no phospholipid neutralizing effect by ORG 25969 like lupus anticoagulant. This, however, does not exclude any possible role of phospholipids in the ORG 25969 anticoagulant effects. It should be noted that both intrinsic tenase and prothrombinase assemble on a phospholipid membrane. In addition, extrinsically activated and RVV-X induced factor Xa generation occur independently of phospholipids and no effect of ORG 25969 on these assays was observed. The potential role of phospholipids should be further addressed by for instance evaluating the effect of ORG 25969 on factor X lacking the γ-carboxyglutamic acid side chains (GIa-domain) responsible for phospholipid interaction. Furthermore, our hypothesized MoA of ORG 25969 by which it interferes with coagulation should be further evaluated in assays employed with purified components. In addition, as indicated earlier, taking along coagulation inhibitors with a known MoA would be important for benchmarking observed ORG 25969 effects.

Conclusion

In summary, the key component of sugammadex, ORG 25969, is responsible for sugammadex-induced APTT and PT prolongations. Adapted, rather than standard, clotting assays were required to shed light on the underlying MoA. Based on this experimental approach, we hypothesize that ORG 25969 may inhibit factor Xa activity in the common pathway and activation of factor X specifically in the intrinsic pathway. Additional research should be performed to confirm this MoA.

REFERENCES

1 P.J. de Kam, P. Grobara, M. Prohn, F. Höppener, *et al., Effects of sugammadex on activated partial thromboplastin time and prothrombin time in healthy subjects.* Int J Clin Pharmacol Ther, 2014. 52(3): p. 227-236.

2 N. Rahe-Meyer, H. Fennema, S. Schulman, W. Klimscha, *et al., Effect of reversal of neuromuscular blockade with sugammadex versus usual care on bleeding risk in a randomized study of surgical patients.* Anesthesiology, 2014. 121(5): p. 969-977.

3 Merck Sharp & Dohme Corp. *Label - Prescribing Information Bridion (sugammadex) Injection, for intravenous use*. 2015; Available from: https://www.accessdata.fda.gov/drugsatfda_docs/label/2015/022225lbl.pdf.

4 C. Kluft, P. Meijer, R. Kret, and J. Burggraaf, *Preincubation in the Prothrombinase-induced Clotting Time test (*PiCT*) is necessary for in vitro evaluation of fondaparinux and to be avoided for the reversible, direct factor Xa inhibitor, rivaroxaban.* Int J Lab. Hematol, 2013. 35(4): p. 379-384.

5 T. Hara, A. Yokoyama, Y. Morishima, and S. Kunitada, *Species differences in anticoagulant and anti-Xa activity of DX-9065a, a highly selective factor Xa inhibitor.* Thromb Res, 1995. 80(1): p. 99-104.

6 E. Perzborn, J. Strassburger, A. Wilmen, J. Pohlmann, *et al., In vitro and in vivo studies of the novel antithrombotic agent BAY 59-7939 - an oral, direct factor Xa inhibitor.* J Thromb Haemost, 2005. 3(3): p. 514-521.

7 P.C. Wong, E.J. Crain, B. Xin, R.R. Wexler, *et al., Apixaban, an oral, direct and highly selective factor Xa inhibitor: in vitro, antithrombotic and antihemostatic studies.* J Thromb Haemost, 2008. 6(5): p. 820-829.

8 P.C. Wong, D.J. Pinto, and R.M. Knabb, *Nonpeptide factor Xa inhibitors: DPC423, a highly potent and orally bioavailable pyrazole antithrombotic agent.* Cardiovasc. Drug Rev, 2002. 20(2): p. 137-152.

9 J.M. Locke, T.K. Stutchbury, K.L. Vine, A.B. Gamble, *et al., Development and assessment of novel all-in-one parenteral formulations with integrated anticoagulant properties for the concomitant delivery of 5-fluorouracil and calcium folinate.* Anticancer Drugs, 2009. 20(9): p. 822-831.

10 T. Moriya, K. Saito, H. Kurita, K. Matsumoto, *et al., A new candidate for an anti-HIV-1 agent: modified cyclodextrin sulfate (mCDS71).* J Med Chem, 1993. 36(11): p. 1674-1677.

11 R.A. Rajewski, G. Traiger, J. Bresnahan, P. Jaberaboansari, *et al., Preliminary safety evaluation of parenterally administered sulfoalkyl ether β-cyclodextrin derivatives.* J Pharm Sci, 1995. 84(8): p. 927-932.

12 C.F. Reilly, T. Fujita, R.C. McFall, I.I. Stabilito, *et al., Pharmacological and mechanistic aspects concerning the use of heparin and β-cyclodextrin tetradecasulfate for the treatment of vascular restenosis.* Drug Dev Res, 1993. 29(2): p. 137-147.

13 K. Shiotani, T. Irie, K. Uekama, and Y. Ishimaru, *Cyclodextrin sulfates in parenteral use: protection against gentamicin nephrotoxicity in the rat.* Eur. J. Pharm. Sci., 1995. 3(3): p. 139-151.

14 J.M. Adam, D.J. Bennett, A. Bom, J.K. Clark, *et al., Cyclodextrin-derived host molecules as reversal agents for the neuromuscular blocker rocuronium bromide: synthesis and structure-activity relationships.* J Med Chem, 2002. 45(9): p. 1806-1816.

15 D.M. Tollefsen, C.A. Pestka, and W.J. Monafo, *Activation of heparin cofactor II by dermatan sulfate.* J Biol Chem, 1983. 258(11): p. 6713-6716.

16 L. Jin, J.P. Abrahams, R. Skinner, M. Petitou, *et al., The anticoagulant activation of antithrombin by heparin.* Proc Natl Acad Sci USA, 1997. 94(26): p. 14683-14688.

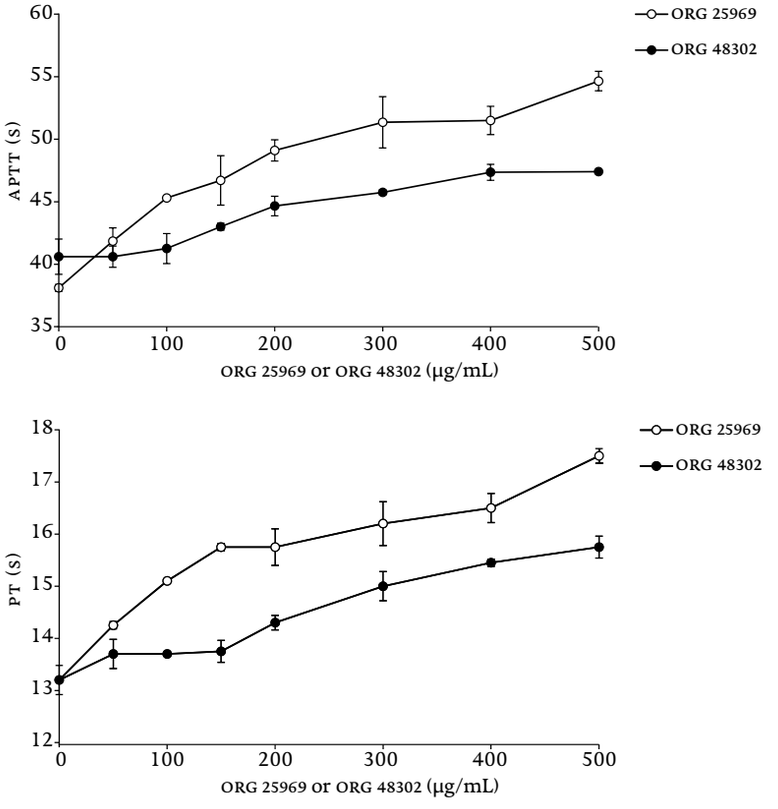
17 F.C. Church, R.E. Treanor, G.B. Sherrill, and H.C. Whinna, *Carboxylate polyanions accelerate inhibition of thrombin by heparin cofactor II.* Biochem Biophys. Res Commun, 1987. 148(1): p. 362-368.

18 N.S. Colwell, M.J. Grupe, and D.M. Tollefsen, *Amino acid residues of heparin cofactor II required for stimulation of thrombin inhibition by sulphated polyanions.* Biochim. Biophys. Acta, 1999. 1431(1): p. 148-156.

19 C.W. Pratt, H.C. Whinna, J.B. Meade, R.E. Treanor, *et al., Physicochemical aspects of heparin cofactor II.* Ann NY Acad Sci, 1989. 556(1): p. 104-115.

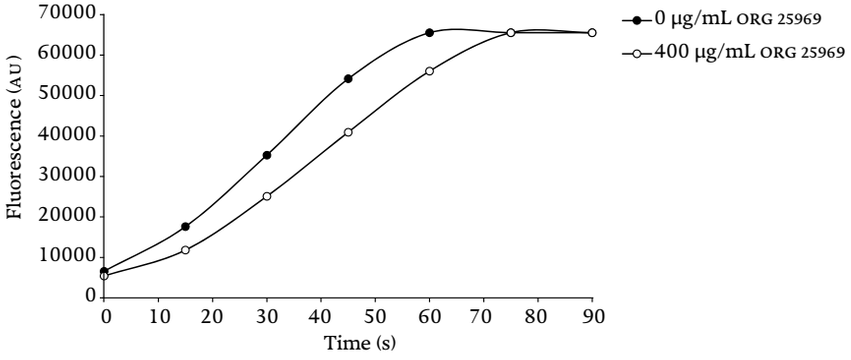
20 D. Dirkmann, M.W. Britten, H. Pauling, J. Weidle, *et al., Anticoagulant effect of sugammadex: just an in vitro artifact.* Anesthesiology, 2016. 124(6): p. 1277-1285.

Figure 1 Mean (±SD of duplicate measurement) effect of ORG 25969 and ORG 48302 on (A) APTT and (B) PT in normal plasma.



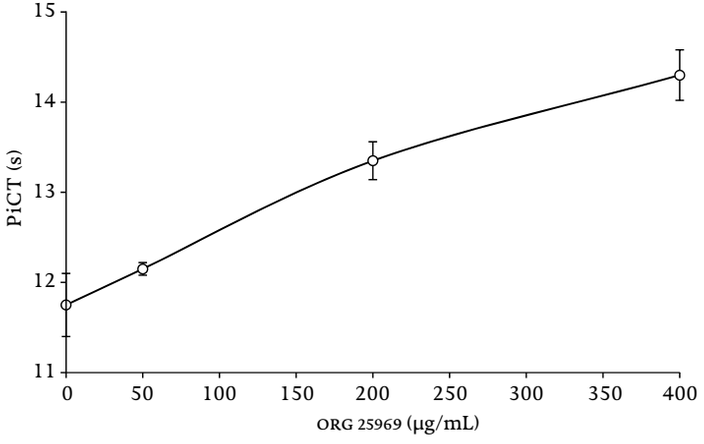
APTT = activated partial thromboplastin time; PT = prothrombin time; and SD = standard deviation.

Figure 2 Representative time course of fluorescence intensity as a marker of thrombin generation, in the presence and absence of 400 µg/mL ORG 25969 in normal plasma. Thrombin generation was activated with PT reagent Innovin. Fluorescence originated from thrombin-induced cleavage of a fluorogenic thrombin substrate and was expressed in arbitrary units (AU).



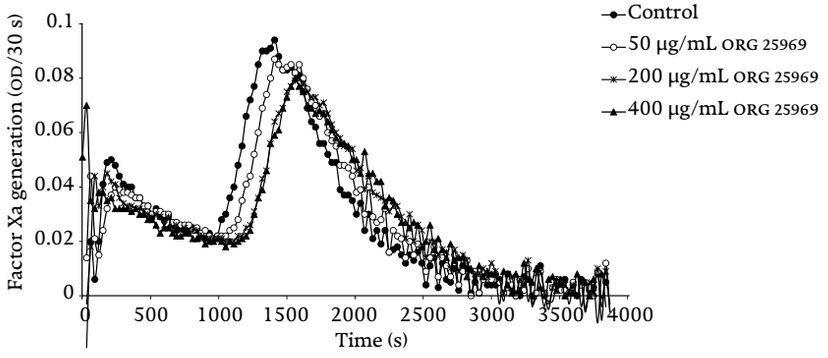
AU = arbitrary units; and PT = prothrombin time.

Figure 3 Mean effect (±SD, duplicate measurement) of ORG 25969 on human one-step PiCT assay in normal plasma.



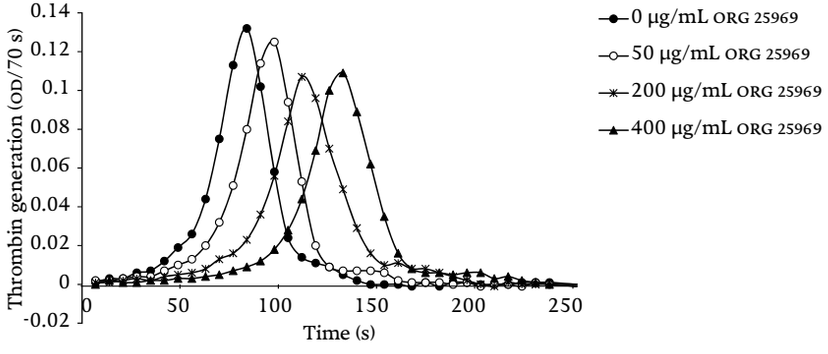
PiCT = prothrombinase-induced clotting time; and SD = standard deviation.

Figure 4 Mean time course (duplicate measurement, coefficient of variation ≤ 11%) of the effect of ORG 25969 on factor Xa generation induced with automated APTT reagent in normal plasma. Factor Xa generation is shown as the first derivative of the optical density (OD) signal which originated from factor Xa-induced cleavage of a chromogenic factor Xa substrate and was assessed every 30 seconds.



APTT = activated partial thromboplastin time; and OD = optical density.

Figure 5 Representative time course of the effect of ORG 25969 on thrombin generation induced with human factor Xa (1%) and rabbit brain cephalin in normal plasma. Thrombin generation is shown as the first derivative of the optical density (OD) signal which originated from thrombin-induced cleavage a chromogenic thrombin substrate and was assessed every 7 seconds.



OD = optical density.

CHAPTER 3

Interactions of sugammadex with various anticoagulants

International Journal of Clinical Pharmacology and Therapeutics, 2020. 58(7)): p. 395-403, doi: 10.5414/CP203733

Annelieke C. Kruithof, Cornelis Kluft; Pieter-Jan de Kam, Geertruida H. Laterveer, Matthijs Moerland, and Jacobus Burggraaf

Abstract

OBJECTIVE To investigate *in vitro* the effect of sugammadex on activated partial thromboplastin time (APTT) and prothrombin time (PT) prolongations with various anticoagulants as well as the neutralizing effect of rocuronium and vecuronium on sugammadex effects on APTT and PT.

MATERIALS AND METHODS We investigated *in vitro* the effect of sugammadex on APTT and/or PT in plasma of patients on a vitamin K antagonist and with elevated international normalized ratios (INRs), in plasma of healthy subjects spiked with either a low or high concentration of enoxaparin, fondaparinux, rivaroxaban, and dabigatran, and in perioperatively collected patient plasma. In addition, we explored whether the effects of sugammadex persisted in the presence of rocuronium or vecuronium, or whether they were counteracted by these compounds.

RESULTS Sugammadex concentration-dependently increased APTT and PT(INR) at all anticoagulant conditions, mainly in a proportional manner, with no differences between perioperatively collected patient and control plasma. Rocuronium and vecuronium both neutralized the effects of sugammadex on APTT and PT.

CONCLUSION Sugammadex has a transient effect on coagulation and is unlikely to increase bleeding risk, this possibility cannot be excluded for scenarios not clinically studied.

Introduction

Sugammadex (Bridion®, MSD, Oss, The Netherlands) is a modified γ-cyclodextrin designed to selectively encapsulate the steroidal neuromuscular blocking agents (NMBAs) rocuronium and vecuronium, thereby reversing the neuromuscular blockade induced by these agents. The clinical application consists of post-operative administration of 2- and 4-mg/kg doses of sugammadex for routine reversal of moderate and deep block, respectively, and 16 mg/kg sugammadex for reversal 3 minutes after an intubating dose of 1.2 mg/kg rocuronium.[1–3]

A previous study in healthy subjects who did not receive an NMBA showed that sugammadex is associated with a limited and transient prolongation of activated partial thromboplastin time (APTT) and international normalized ratio for prothrombin time PT(INR). These effects were short lasting (≤ 30 minutes) and dose-dependent, with an 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. The effects of sugammadex on APTT and PT(INR) may be explained by a decrease in factor Xa activity in the common pathway and in the formation of factor Xa specifically in the intrinsic pathway.[4] The limited effect of sugammadex on coagulation was also noted in patients receiving commonly prescribed prophylactic antithrombotic therapy and undergoing major orthopedic surgery, where it was shown that sugammadex (4 mg/kg) caused limited (< 8%), transient (< 1 hour) APTT and PT(INR) increases in patients as compared to usual care (neostigmine or spontaneous recovery),[5] comparable to the increases observed in healthy subjects.[4] Importantly, the primary results of this dedicated study in at-risk patients demonstrated that the use of sugammadex was not associated with an increased risk of bleeding complications.[5]

The potential interactions between sugammadex and anticoagulants were assessed in healthy subjects and patients where thromboprophylaxis was limited to low-molecular-weight heparin (LMWH), unfractionated heparin (UFH), and/or aspirin at relatively low doses of the anticoagulant/antiplatelet agents.[5–7] However, it is unknown whether sugammadex interacts with classical anticoagulants at high dose levels. Moreover, no information is available on the potential interaction between sugammadex and novel oral anticoagulants, and on the possible effect of NMBAs on such an interaction.

Therefore, we investigated *in vitro* the effects of sugammadex on APTT and/or PT(INR) in plasma with elevated INRs as well as in plasma with either a low or high level of the classical agents enoxaparin and fondaparinux and the novel agents rivaroxaban and dabigatran, and in perioperatively collected patient plasma. Furthermore, we scrutinized whether the effects of sugammadex remain unaffected by the presence of rocuronium or vecuronium or are counteracted by an NMBA. As we know from previous research that the coagulation effects of sugammadex are mainly caused by the modified γ-cyclodextrin ORG 25969 (data on file), the primarily component of sugammadex (> 93%),[2] we only included ORG 25969 in the present experiments.

Methods and materials

All experiments were performed by the Centre for Human Drug Research (CHDR, Leiden, The Netherlands) at the laboratories of Good Biomarker Sciences (GBS, Leiden, The Netherlands).

Blood from 12 consenting, apparently healthy subjects was collected in citrated (0.105 M) tubes and centrifuged twice for 20 minutes at 2,000 × g at 4 °C. After pooling plasma of 6 subjects, aliquots were stored at –60 °C and thawed only once prior to use. Plasma was spiked with a low and high concentration of enoxaparin (Aventis, Sanofi Aventis, Gouda, The Netherlands), fondaparinux (GlaxoSmithKline BV, Zeist, The Netherlands), rivaroxaban (Tanabe Seiyaku Co., Ltd., Osaka, Japan) or dabigatran (Schering-Plough, Newhouse, United Kingdom) to cover both ends of the therapeutic range of these compounds. In addition to plasma from apparently healthy subjects, plasma from 3 vitamin K antagonist-treated patients with INRs of 2.3, 3.7 and 4.4 were obtained from the Dutch Thrombosis Service. Patients in the Netherlands treated with either acenocoumarol or phenprocoumon report regularly to the Thrombosis Service for monitoring of their anticoagulant therapy. Besides this, plasma from 8 patients in the perioperative period of cardiac surgery was obtained from a hospital. Lastly, pooled plasma from apparently healthy subjects was spiked with rocuronium (10 mg/mL) or vecuronium (10 mg for 5 mL), which was both obtained from Organon, Oss, The Netherlands.

In all plasma conditions, the effects of ORG 25969 (laboratory code of sugammadex), which was kindly provided by Organon (Oss, The Netherlands), was investigated. ORG 25969 is the main active component of sugammadex (> 93%)[2] and primarily contributory to the sugammadex effects on APTT and PT(INR) (data on file). Concentrations of 50 and 200 µg/mL ORG 25969 were chosen to resemble the peak plasma concentrations (C_{max}) following clinical doses of 4 and 16 mg/kg for routine and immediate reversal, respectively. In addition, the supratherapeutic concentration 400 µg/mL ORG 25969 was explored to further elucidate the effects of ORG 25969 on coagulation. In all control and ORG 25969-spiked plasma samples, APTT and/or PT(INR) was assessed. A commercially available APTT kit (bioMérieux, Marcy l'Etoille, France) was used to measure APTT on the automated clotting analyzer AMAX Destiny Plus (Kordia Lifesciences, Leiden, The Netherlands). 25 µL of plasma was pre-incubated with 25 µL automated APTT reagent-containing rabbit brain phospholipids and microsilica activator followed by the addition of 25 µL of calcium chloride solution to induce clotting. PT(INR) was assessed by mixing 25 µL of plasma with 50 µL Simplastin HTF reagent containing human tissue factor thromboplastin derived from cultured human cells and calcium ions (bioMérieux, Marcy l'Etoille, France) on the AMAX Destiny Plus. We are aware that INR is the common read-out for the effects of vitamin K antagonist, however, for reasons of graphical presentation, we chose to present the data as PT, which give similar results as the INR. All measurements were performed in duplicate using two aliquots from the same plasma donor/pool.

Results

HIGH INR PLASMA

The effects of ORG 25969 on clotting times in plasma from vitamin K antagonist-treated patients with INRs of 2.3, 3.7 and 4.4 were investigated and compared to pooled plasma from apparently healthy subjects with an INR of 1.0 (Figure 1). ORG 25969 induced concentration-dependent increases in PT(INR) and APTT in all INR conditions. In general, these prolongations were INR-dependent and most pronounced at an INR of 4.4. The addition of 50 µg/mL ORG 25969 resulted in INR increases of 0.2–0.6 (PT: 2.3–5.0 seconds) and 0.1 (PT: 1.1 second) in patients and control plasma, respectively. Raising the ORG 25969 concentration to 400 µg/mL ORG 25969 generated increases of 1.1–2.1 INR units (PT: 10.9–18.2 seconds) in patients' plasmas and 0.3 INR unit (PT: 3.3 seconds) in control plasma.

ANTICOAGULANTS

The effects of various concentrations of ORG 25969 on APTT of normal plasma and plasma spiked with a low or high concentration of enoxaparin, fondaparinux, rivaroxaban, or dabigatran are displayed in Figure 1. Increasing ORG 25969 concentration up to 400 µg/mL induced higher APTT values at both low and high concentrations of the investigated anticoagulants. The APTT prolongations were most distinct for the low level of dabigatran (106 ng/mL) and the high level of rivaroxaban (200 ng/mL) in terms of both absolute and relative change. The latter condition had APTTs of 56.7 and 101.8 seconds in the absence and presence of 400 µg/mL ORG 25969, respectively, compared to 39.3 and 56.2 seconds in control plasma.

PERIOPERATIVE SAMPLES

The effects of ORG 25969, up to 400 µg/mL, were not different in plasma collected from patients in the perioperative period with a PT ranging from 13.0 to 21.2 seconds and in control plasma with a PT of 13.4 seconds (Figure 1). Likewise, APTT prolongation by ORG 25969 in these plasmas with an APTT ranging from 34.6 to 56.1 seconds was comparable to control plasma with an APTT of 41.1 seconds.

INFLUENCE OF NMBAS

The potential modulatory role of NMBAs on ORG 25969 effects on APTT and PT was investigated in pooled plasma from apparently healthy subjects (Figure 2 and Figure 3, respectively). The effect of ORG 25969 on APTT and PT decreased with increasing concentrations of both vecuronium and rocuronium. The effects were completely neutralized when equimolar amounts of rocuronium and ORG 25969 were present. A similar trend for vecuronium was observed, but concentrations equimolar to 200 and 400 µg/mL ORG 25969 could not be assessed due to the dilution of the stock.

Discussion

Sugammadex reverses neuromuscular blockades by encapsulation of the NMBAs rocuronium or vecuronium. It has an effect on coagulation as manifested by limited and transient APTT and PT(INR) prolongations without known bleeding complications in healthy subjects pretreated with nothing,[4] aspirin,[6] enoxaparin, or UFH[7] and surgical patients receiving thromboprophylaxis (mainly LWMH) and undergoing major orthopedic surgery.[5]

The present *in vitro* investigation was designed to explore potential interactions between sugammadex and various anticoagulant conditions beyond the ones evaluated in clinical studies such as novel direct oral agents and high levels of classical anticoagulants. The active drug substance of sugammadex consists primarily of ORG 25969.[2] As ORG 25969 is mainly responsible for the sugammadex effects on APTT and PT(INR) (data on file), we only assessed ORG 25969. APTT, the most sensitive coagulation test for *in vitro* sugammadex effects,[4] was selected as read-out measure. In case a vitamin K antagonist could be present, PT(INR) was also assessed as INR guides vitamin K antagonist dose adjustments. We used the APTT and PT(INR) assay kits that were most sensitive to ORG 25969 (data on file). ORG 25969 was added up to 400 µg/mL, covering concentrations of 50 and 200 µg/mL that represent the mean C_{max} following clinical doses of 4 and 16 mg/kg sugammadex, respectively. The highest clinical dose is only given to achieve immediate reversal in emergency situations (*i.e.*, 3 minutes after a dose of 1.2 mg/kg rocuronium),[2,3] which rarely occurs.[8–10] In such potentially life-threatening situation, the benefit of sugammadex clearly outweighs the potentially associated induced APTT/PT increases, especially considering that generally surgery has not been initiated yet and is likely to be postponed if possible. Nevertheless, concentrations above 50 µg/mL were investigated to provide further insights in the effects of sugammadex on coagulation.

We found that ORG 25969 induced concentration-dependent PT(INR) and APTT prolongations in plasma from patients on vitamin K antagonists with elevated INRs over a range of 2.3–4.4 analogue to control plasma with an INR of 1.0, with generally more pronounced increases at higher INRs. These increases seem to be proportional, in particular at lower INRs.

Next we investigated the possible interaction between ORG 25969 and classical indirect and novel direct inhibitors of factor Xa and/or thrombin. Enoxaparin and fondaparinux were chosen to represent the indirect agents, and dabigatran and rivaroxaban to represent direct agents. Plasma was incubated with a low and high concentration of each anticoagulant to cover both ends of their therapeutic window. We found that ORG 25969 concentration-dependently prolonged APTT in all conditions, with the strongest increases recorded for the low level of thrombin inhibitor dabigatran (106 ng/mL) and the high level of factor Xa inhibitor rivaroxaban (200 ng/mL). Occasionally,

raising the concentration of ORG 25969 resulted in a relatively smaller APTT increase, which we consider to be spurious. We previously observed comparable concentration-effect relationships of ORG 25969 on APTT and PT in antithrombin deficient and control plasma.[4] Hence, strong potentiating of ORG 25969 effects by antithrombin-dependent factor Xa and/or thrombin inhibitors enoxaparin and fondaparinux were also not expected. Further, a pharmacokinetic-pharmacodynamic (PK-PD) model of clinical sugammadex effects on APTT and PT(INR) suggested a maximum effect rather than a linear relationship, especially at concentrations above 150 μg/mL.[11] This model relates to our control and low level of enoxaparin conditions. Although our data are limited, they seem to corroborate the findings of the PK-PD model for the control condition, while for enoxaparin a maximum effect seems to appear at a high concentration.

To facilitate translation of the experimental setting into clinical practice, the effect of ORG 25969 in plasma obtained from patients in the perioperative setting of cardiac surgery was compared with control plasma. ORG 25969 up to a supratherapeutic concentration of 400 μg/mL elicited a similar pattern of prolongation over a wide range of APTT and PT in patient samples and in control plasma. We also explored the potential modulatory role of the NMBAs rocuronium and vecuronium on the effect of ORG 25969 on APTT and PT. This effect was lower in the presence of an NMBA. Moreover, no effect was found at equimolar concentrations of ORG 25969 and rocuronium, which is line with a previous report.[12] In the clinical practice, both ORG 25969 and an NMBA are present, so the actual free ORG 25969 fraction available for coagulation interference in the clinical setting is likely lower than we applied in our *in vitro* experiments.

Two limitations of our study may affect the translation of our findings into clinical practice. First of all, both APTT and PT(INR) are surrogate coagulation markers rather than predictive biomarkers for clinical outcome such as bleeding risk. Moreover, spiking plasma with enoxaparin results in higher APTTs compared to values from the same subjects treated with enoxaparin (data on file). This implies that spiking experiments may slightly overestimate the actual effect on the test and the possible clinical effect. Unfortunately, real-life benchmark data to link APTT and PT(INR) prolongations and bleeding risk for most anticoagulants are scarce, especially for short-lasting (< 1 hour) prolongations as observed with sugammadex.[4–7] Prolonged increases have been investigated with a focus on long-lived (months-years) elevated INRs. This has clearly revealed that high INRs are a major determinant of bleeding risk.[13–16] However, this does not necessarily apply to more short-lived INR increases in the perioperative setting. For instance, Torn *et al.* reported that the level of anticoagulation on the day of surgery was not key to the occurrence of bleeding for patients on chronic vitamin K antagonist therapy with an inadequate INR prior to routine surgery, including major surgery.[17] This suggests that both extent and duration of an INR increase contribute to bleeding risk. The limited predictability of preoperative increased PT and APTT for post-operative bleeding has also been reported by others.[18–20] Furthermore, inconclusive correlations were obtained for coagulation values in the immediate post-operative setting,[21,22] suggesting that bleeding risk in the perioperative setting is not predominantly coagulation-derangement driven. Therefore, our *in vitro* results cannot be directly translated into bleeding risk. Nonetheless, the effect of LMWH and sugammadex combined was already investigated in healthy subjects and in a dedicated bleeding study in surgical patients; both showed no coagulation/bleeding complications.[5,7] Our *in vitro* experiments did not reveal major differences in effect size when taking enoxaparin as reference point. Nevertheless, the different classes of anticoagulants are not interchangeable, and the *in vitro* enoxaparin concentrations were higher than the clinical concentrations just prior sugammadex administration (data on file).

Alternative benchmark data could be within-patient variation in APTT and PT(INR). However, this has only reported by a few studies that focused on vitamin K antagonist-treated patients. The noted approximate diurnal variation of 5–10%[23–25] appears to concur with the INR increase induced by 50 μg/mL ORG 25969 (corresponding with 4 mg/kg sugammadex) in plasma with elevated INRs, especially for INR ≤ 3.7.

Perioperative management of the investigated anticoagulants should also be taken into account. All are indicated for post-operative thromboprophylaxis following abdominal and/or orthopedic surgery. Providing that hemostasis has been achieved, enoxaparin, fondaparinux, rivaroxaban, and dabigatran should be in general initiated 12–24[26], 6[27]–8[28], 6–10[29,30] and 1–4[31,32] hours post-surgery, respectively, according to their labels. As the anticoagulant effect of sugammadex is short-lasting (< 1 hour), it is unlikely that sugammadex will augment the effectiveness of these therapies. Thromboprophylaxis may also be initiated preoperatively in patients at high risk for thromboembolic complications.[26] Such routinely prescribed antithrombotic therapy, commonly

LMWH, did not predispose patients undergoing major orthopedic surgery to additional bleeding risk when receiving 4 mg/kg sugammadex in comparison with usual care.[5] In patients at risk of bleeding, anticoagulant therapy is normally temporarily discontinued prior to elective surgery. However, clinically relevant anticoagulant levels may be present when non-elective surgery is anticipated. A reversal agent, if available, could then be administered such as vitamin K for vitamin K antagonist therapy[33,34] and idarucizumab for dabigatran.[35] For heparin-based anticoagulants, only protamine sulfate is available, which neutralizes heparin, but seems only be partially able to neutralize LMWH as suggested by sparse clinical data and is unable to neutralize fondaparinux.[36–38] For rivaroxaban, andexanet alfa became recently available in the United States of America and is currently under review in the European Union.[39] Nevertheless, it may occur in rare instances such as emergency surgery that sugammadex is administered to patients who have antithrombotic prophylactic levels beyond the ones clinically studied.[5] Physicians should be aware that an increased bleeding risk cannot be excluded in such cases.

Conclusion

In summary, ORG 25969 (sugammadex) concentration-dependently prolonged APTT and PT(INR) in the presence of vitamin K antagonists enoxaparin, fondaparinux, dabigatran, and rivaroxaban. The effects of sugammadex on APTT and PT were counteracted by rocuronium and vecuronium, so the actual free sugammadex fraction available for coagulation interference in the clinical setting is likely lower than studied in these *in vitro* experiments.

REFERENCES

1 S.J. Schaller and H. Lewald, *Clinical pharmacology and efficacy of sugammadex in the reversal of neuromuscular blockade.* Expert Opin Drug Metab Toxicol, 2016. 12(9): p. 1097-1108.

2 Merck Sharp & Dohme Corp. *Label - Prescribing Information Bridion (sugammadex) Injection, for intravenous use*. 2015; Available from: https://www.accessdata.fda.gov/drugsatfda_docs/label/2015/022225lbl.pdf.

3 Merck Sharp & Dohme Limited. *Bridion Annex I: Summary of Product Characteristics*. 2018; Available from: https://www.ema.europa.eu/documents/product-information/bridion-epar-product-information_en.pdf.

4 P.J. de Kam, P. Grobara, M. Prohn, F. Höppener, *et al.*, *Effects of sugammadex on activated partial thromboplastin time and prothrombin time in healthy subjects.* Int J Clin Pharmacol Ther, 2014. 52(3): p. 227-236.

5 N. Rahe-Meyer, H. Fennema, S. Schulman, W. Klimscha, *et al.*, *Effect of reversal of neuromuscular blockade with sugammadex versus usual care on bleeding risk in a randomized study of surgical patients.* Anesthesiology, 2014. 121(5): p. 969-977.

6 P.J. de Kam, R. El Galta, A.C. Kruithof, H. Fennema, *et al.*, *No clinically relevant interaction between sugammadex and aspirin on platelet aggregation and coagulation parameters.* Int J Clin Pharmacol Ther, 2013. 51(12): p. 976-985.

7 P.J. de Kam, A.C. Kruithof, M.J. van Lierop, M. Moerland, *et al.*, *Lack of a clinically relevant effect of sugammadex on anti-Xa activity or activated partial thromboplastin time following pretreatment with either unfractionated or low-molecular-weight heparin in healthy subjects.* Int J Clin Pharmacol Ther, 2014. 52(8): p. 631-641.

8 T.M. Cook, N. Woodall, C. Frerk, and Fourth National Audit Project, *Major complications of airway management in the UK: results of the Fourth National Audit Project of the Royal College of Anaesthetists and the Difficult Airway Society. Part 1: anaesthesia.* Br J Anaesth, 2011. 106(5): p. 617-631.

9 C. Frerk, V.S. Mitchell, A.F. McNarry, C. Mendonca, *et al.*, *Difficult Airway Society 2015 guidelines for management of unanticipated difficult intubation in adults.* Br J Anaesth, 2015. 115(6): p. 827-848.

10 W.J. Herring, T. Woo, C.A. Assaid, R.J. Lupinacci, *et al.*, *Sugammadex efficacy for reversal of rocuronium- and vecuronium-induced neuromuscular blockade: a pooled analysis of 26 studies.* J Clin Anesth, 2017. 41: p. 84-91.

11 R. Bosch, M.J. van Lierop, P.J. de Kam, A.C. Kruithof, *et al.*, *A PK-PD model-based assessment of sugammadex effects on coagulation parameters.* Eur J Pharm Sci, 2016. 84: p. 9-17.

12 D. Dirkmann, M.W. Britten, H. Pauling, J. Weidle, *et al.*, *Anticoagulant effect of sugammadex: just an in vitro artifact.* Anesthesiology, 2016. 124(6): p. 1277-1285.

13 R.G. Hart, B.S. Boop, and D.C. Anderson, *Oral anticoagulants and intracranial hemorrhage. Facts and hypotheses.* Stroke, 1995. 26(8): p. 1471-1477.

14 J. Hirsh, J. Dalen, D.R. Anderson, L. Poller, *et al.*, *Oral anticoagulants: mechanism of action, clinical effectiveness, and optimal therapeutic range.* Chest, 2001. 119(1 Suppl): p. 8S-21S.

15 M.N. Levine, G. Raskob, R.J. Beyth, C. Kearon, *et al.*, *Hemorrhagic complications of anticoagulant treatment: the Seventh ACCP Conference on Antithrombotic and Thrombolytic Therapy.* Chest, 2004. 126(3 Suppl): p. 287S-310S.

16 G. Palareti, N. Leali, S. Coccheri, M. Poggi, *et al.*, *Bleeding complications of oral anticoagulant treatment: an inception-cohort, prospective collaborative study (ISCOAT).* Lancet, 1996. 348(9025): p. 423-428.

17 M. Torn and F.R. Rosendaal, *Oral anticoagulation in surgical procedures: risks and recommendations.* Br J Haematol, 2003. 123(4): p. 676-682.

18 T. Bombeli and D.R. Spahn, *Updates in perioperative coagulation: physiology and management of thromboembolism and haemorrhage.* Br J Anaesth, 2004. 93(2): p. 275-287.

19 M.J. Rohrer, M.C. Michelotti, and D.L. Nahrwold, *A prospective evaluation of the efficacy of preoperative coagulation testing.* Ann Surg, 1988. 208(5): p. 554-557.

20 A.L. Suchman and A.I. Mushlin, *How well does the activated partial thromboplastin time predict postoperative hemorrhage?* JAMA, 1986. 256(6): p. 750-753.

21 A.B. Gelb, R.I. Roth, J. Levin, M.J. London, *et al.*, *Changes in blood coagulation during and following cardiopulmonary bypass: lack of correlation with clinical bleeding.* Am J Clin Pathol, 1996. 106(1): p. 87-99.

22 G.A. Nuttall, W.C. Oliver, M.H. Ereth, and P.J. Santrach, *Coagulation tests predict bleeding after cardiopulmonary bypass.* J Cardiothorac Vasc Anesth, 1997. 11(7): p. 815-823.

23 B.E. Bleske, L.S. Welage, E.W. Warren, M.B. Brown, *et al.*, *Variations in prothrombin time and international normalized ratio over 24 hours in warfarin-treated patients.* Pharmacotherapy, 1995. 15(6): p. 709-712.

24 C.H. Ho, M.W. Lin, J.Y. You, C.C. Chen, *et al.*, *Variations of prothrombin time and international normalized ratio in patients treated with warfarin.* Thromb Res, 2002. 107(5): p. 277-280.

25 A. García, F. Marín, B. Sánchez, V. Roldán, *et al.*, *Diurnal variation in the intensity of anticoagulation in atrial fibrillation.* Stroke, 2002. 33(1): p. 322-324.

26 Sanofi-Aventis U.S. LLC. *Label - Prescribing Information Lovenox (enoxaparin sodium injection) for subcutaneous and intraveneous use*. 2018; Available from: https://www.accessdata.fda.gov/drugsatfda_docs/label/2018/020164s116lbl.pdf.

27 Aspen Pharma Trading Limited. *Arixtra Annex I: Summary of Product Characteristics*. 2018; Available from: https://www.ema.europa.eu/documents/product-information/arixtra-epar-product-information_en.pdf.

28 Aspen NDB. *Label - Prescribing Information Arixtra (fondaparinux sodium) Solution for subcutaneous injection*. 2017; Available from: https://www.accessdata.fda.gov/drugsatfda_docs//label/2017/021345s035lbl.pdf.

29 Boehringer Ingelheim Pharmaceuticals Inc. *Label – Prescribing Information Pradaxa (dabigatran etexilate mesylate) capsules, for oral use*. 2018; Available from: https://www.accessdata.fda.gov/drugsatfda_docs/label/2018/022512s035lbl.pdf.

30 Boehringer Ingelheim International GmbH. *Pradaxa Annex I: Summary of Product Characteristics*. 2018; Available from: https://www.ema.europa.eu/documents/product-information/pradaxa-epar-product-information_en.pdf.

31 Bayer Pharma AG. *Xarelto Annex I: Summary of Product Characteristics*. 2018; Available from: https://www.ema.europa.eu/documents/product-information/xarelto-epar-product-information_en.pdf.

32 Janssen Pharmaceuticals Inc. *Label – Prescribing Information Xarelto (rivaroxaban) tablets, for oral use*. 2019; Available from: https://www.accessdata.fda.gov/drugsatfda_docs/label/2019/022406s030s032lbledt.pdf.

33 J. Ansell, J. Hirsh, E. Hylek, A. Jacobson, *et al.*, *Pharmacology and management of the vitamin K antagonists: American College of Chest Physicians Evidence-Based Clinical Practice Guidelines (8th Edition).* Chest, 2008. 133(6 Suppl): p. 160S-198S.

34 D. Keeling, T. Baglin, C. Tait, H. Watson, *et al.*, *Guidelines on oral anticoagulation with warfarin - fourth edition.* Br J Haematol, 2011. 154(3): p. 311-324.

35 J.W. Eikelboom, D.J. Quinlan, J. van Ryn, and J.I. Weitz, *Idarucizumab: the antidote for reversal of dabigatran.* Circulation, 2015. 132(25): p. 2412-2422.

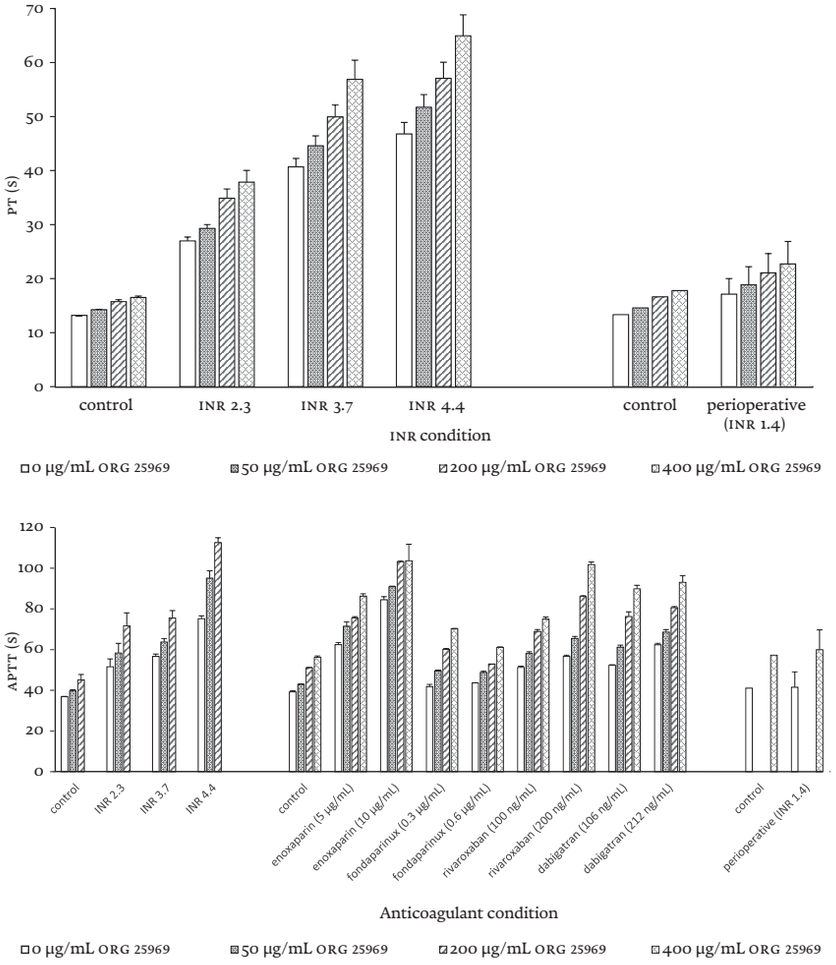
36 J. Hirsh, K.A. Bauer, M.B. Donati, M. Gould, *et al.*, *Parenteral anticoagulants: American College of Chest Physicians Evidence-Based Clinical Practice Guidelines (8th Edition).* Chest, 2008. 133(6 Suppl): p. 141S-159S.

37 M. Makris, J.J. Van Veen, C.R. Tait, A.D. Mumford, *et al.*, *Guideline on the management of bleeding in patients on antithrombotic agents.* Br J Haematol, 2013. 160(1): p. 35-46.

38 M.T. Kalathottukaren, C.A. Haynes, and J.N. Kizhakkedathu, *Approaches to prevent bleeding associated with anticoagulants: current status and recent developments.* Drug Deliv Transl Res, 2018. 8(4): p. 928-944.

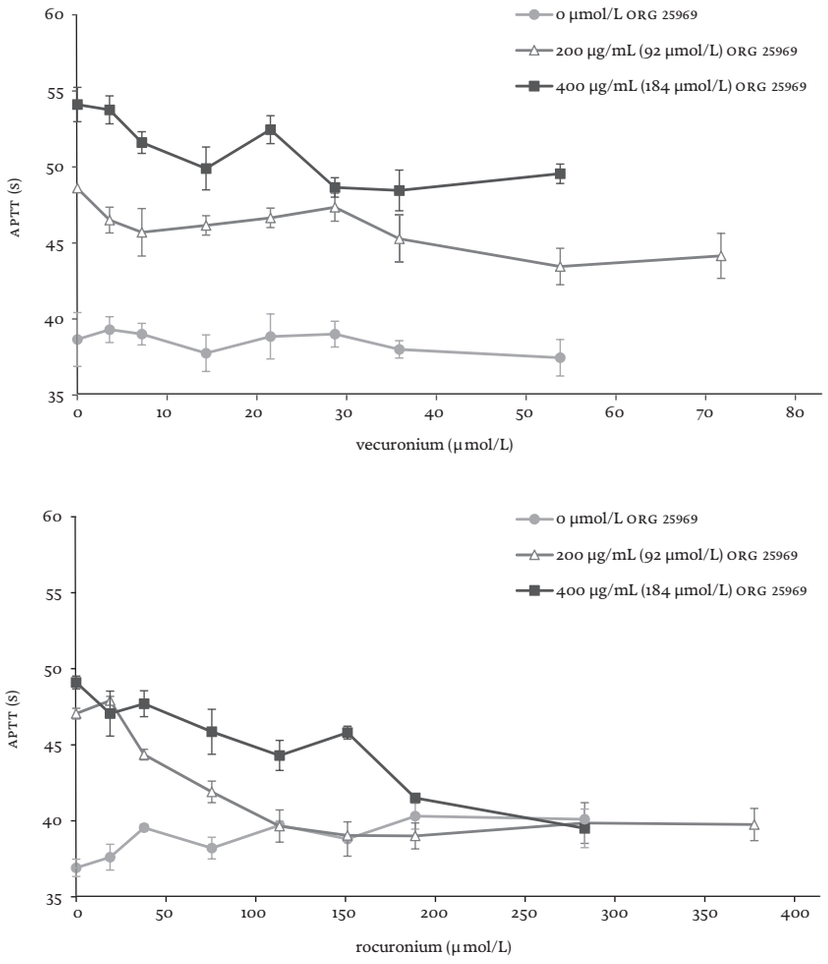
39 Y.A. Heo, *Andexanet alfa: first global approval.* Drugs, 2018. 78(10): p. 1049-1055.

Figure 1 Mean effects of ORG 25969 on (A) PT and (B) APTT in plasma from vitamin K antagonist-treated patients with elevated INRs, anticoagulant-spiked plasma, plasma from patients in perioperative setting, and corresponding control plasma. Error bars represent standard deviation with n = 2 for all conditions except perioperative plasma with n = 8.



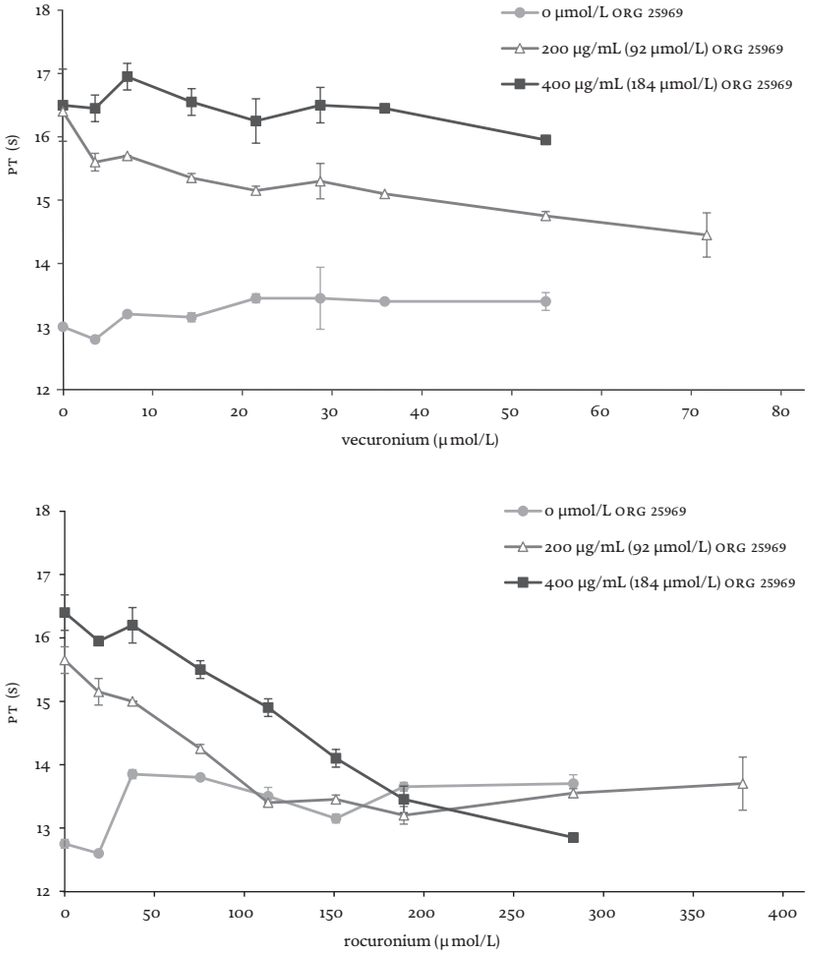
APTT = activated partial thromboplastin time; INR = international normalized ratio; and PT = prothrombin time

Figure 2 Mean effect of (A) vecuronium and (B) rocuronium on APTT in the absence and presence of ORG 25969 in normal plasma. The condition with 200 µg/mL (92 µmol/L) ORG 25969 was tested separately. Error bars represent standard deviation, n = 2.



APTT = activated partial thromboplastin time.

Figure 3 Mean effect of (A) vecuronium and (B) rocuronium on PT in the absence and presence of ORG 25969 in normal plasma. The condition with 200 µg/mL (92 µmol/L) ORG 25969 was tested separately. Error bars represent standard deviation, n = 2.



PT = prothrombin time.

CHAPTER 4

Evaluation of the effect of aspirin on platelet aggregation: methodological recommendations for aspirin-drug interaction studies

Journal of Biomedical Science and Engineering, 2015.
8(1): p. 40-45, doi: 10.4236/jbise.2015.81004

Annelieke C. Kruithof, Matthijs Moerland, Eleftheria A. Anastasopoulou,
Pieter-Jan de Kam, Marieke L. de Kam, and Jacobus Burggraaf

Abstract

Given the broad application of aspirin as antiplatelet drug, availability of standardized methodology to assess potential interaction with any co-medication on platelet aggregation is desired. We characterized the effect of aspirin (ASA) therapy on collagen-induced platelet aggregation in whole blood to define such methodology. Collagen-induced platelet whole blood aggregation was assessed in 6 healthy male volunteers on 2 occasions (Day 1, Day 7) using the Chronolog aggregometer. From Day 2 up to Day 7, subjects received a daily oral dose of 75 mg ASA. The relationship between collagen dose and platelet aggregation response was assessed. On Day 1, maximal aggregation was observed at 1 μg/mL collagen (15.3 ±4.6 Ω) and higher. Reproducible results were obtained without any indication of intra-subject fluctuations. ASA treatment decreased maximal aggregation by 80% and 38% at 0.5 and 2.0 μg/mL collagen, respectively. Power calculations were performed based on the observed intra-subject variability and demonstrated minimal sample sizes of 9–11 subjects for future cross-over ASA-drug interaction studies exploring effects on platelet aggregation, which demonstrates that the proposed collagen-induced *ex vivo* whole blood platelet aggregation is a feasible methodology to evaluate ASA-drug interactions in healthy volunteers.

Introduction

Aspirin (acetylsalicylic acid; ASA) is the most widely used platelet function inhibitor.[1] Low ASA doses of 75–325 mg/day are prescribed for prevention of thrombotic cardiovascular disease as recommended by the American College of Chest Physicians (ACCP).[2] The number of patients on long-term ASA treatment receiving a wide variety of concomitant drugs, such as statins and angiotensin-converting-enzyme (ACE) inhibitors, is relatively high.[3] Therefore, availability of standardized methodology evaluating the potential interaction between ASA and any co-medication on platelet function is desired. Ideally, such methodology should represent human physiology, be reproducible, have minimal intra-subject variation, and provide a sufficient window for evaluations of changes in platelet function.

A frequently used method for quantification of the effect of antiplatelet drugs is *ex vivo* platelet aggregation in response to an agonist, so called platelet aggregometry. The preferred matrix for this method is whole blood rather than platelet rich plasma (PRP) since whole blood has no need of sample centrifugation which may introduce artifacts and it more closely resembles the real-life physiologic conditions, due to the presence of red blood cells and other cell types.[4–6] Using whole blood aggregometry, different platelet agonists can be used to assess the inhibitory effect of ASA, either alone or in combination with other anti-aggregant agents, such as arachidonic acid (AA), collagen, adenosine phosphate (ADP) and adrenaline. Generally, 1 relatively high agonist concentration is selected to activate platelets.[7–12] This approach is particularly useful to discriminate between ASA responders and non-responders. However, for studying potential ASA-drug interactions this experimental setup is less feasible since induction of a maximal platelet aggregation level does not allow demonstration of drug-induced increases or subtle decreases in the aggregatory response.

Therefore, we aimed to develop a platelet aggregometry methodology feasible for evaluation of potential interaction between ASA and any drug-of-choice, which should facilitate rational design of ASA-drug interaction studies. For this purpose, we characterized the modulation of whole blood platelet aggregation by ASA therapy. We selected collagen as aggregation inducer since it plays a key role in the initial stage of the haemostatic cascade *in vivo*, *i.e.* the interaction of platelets with the extracellular matrix (ECM) of an injured vascular wall. Collagen is the most abundant thrombogenic component

present in ECM and its interaction with platelets triggers platelet activation and subsequently platelet adhesion and aggregation.[13] Collagen-induced whole blood platelet aggregometry is a well-known method to assess ASA responsiveness, to discriminate between ASA responders and non-responders and to determine efficacy of dual ASA therapy,[14,15] however, it has not been evaluated for assessment of ASA-drug interactions. Therefore, we assessed the relationship between collagen concentration and platelet aggregation response in a relatively small group of healthy male volunteers, since this is a common population for drug-drug interaction studies. Assay reproducibility and intra-subject variability in the aggregatory response were explored to assess method robustness. The effect of ASA treatment on collagen-induced *ex vivo* whole blood platelet aggregation (amplitude, lag time and aggregation rate) was evaluated, with the aim to define the methodological conditions inducing a level of aggregation offering sufficient window for demonstration of the presence or absence of additional effects of any drug-of-choice in an ASA interaction study. Based on the observed variability, power calculations were performed to estimate desired sample sizes for future ASA-drug interaction studies.

Methods and Materials

The study was approved by the Medical Ethics Committee of Leiden University Medical Center, the Netherlands, and conducted in compliance with Dutch law on experiments in humans. All subjects gave written informed consent.

STUDY POPULATION

Six healthy male volunteers aged 18–55 years participated in the study, with a body mass index (BMI) between 18 and 32 kg/m^2 and a normal activated partial thromboplastin time (APTT), prothrombin time (PT) and bleeding time at screening, and no clinically significant findings in (family) medical history (*e.g.* hematological disorders), physical examination and laboratory tests. The use of concomitant medication was prohibited.

STUDY DESIGN

This open-label study was conducted in a study period consisting of 7 days. On Day 1 and Day 7, subjects stayed at the clinical research unit for assessment of platelet aggregation. From the morning of Day 2 until Day 7 (6 consecutive days), subjects visited the clinical research unit daily for supervised oral administration of 75 mg ASA (Actavis®). On Day 1 (before start of ASA treatment) and Day 7 (during ASA treatment), blood samples were taken at −5 minutes, 45 minutes and 1, 1.5, 3.5 and 6.5 hours. T = 0 on Day 1 and Day 7 were comparable actual clock times, but only on t = 0 at Day 7 ASA was administered. Blood was collected in 5.0 mL tubes containing 1/10 citrate solution (Sarstedt, Nümbrecht, Germany) using a free-flow 18 gauge intravenous catheter without venous occlusion (Bection Dickinson, Franklin Lanes, United States of America (USA)) after discarding the first 0.5 mL blood.

PLATELET AGGREGATION

Platelet aggregation was assessed using the Chronolog 590-X whole blood impedance aggregometer (Chrono-Log, Corp., Pennsylvania, USA). Platelet aggregation was induced by rising collagen concentrations (0.5, 0.75, 1 and 2 µg/mL; type 1 fibrils, Chrono-Par reagents, Chrono-Log Corp., stock solution 1 mg/mL, diluted in isotonic glucose solution at pH 2.7–2.9). Measurements of platelet aggregation were performed in duplicate for each collagen concentration. Addition of collagen to whole blood sample corresponded with the start of recording an impedance curve which was allowed to run for 15 minutes or until a constant amplitude have been reached. Aggregometry results were expressed as maximum amplitude (resembling maximal aggregation) and recorded in Ohm (Ω). The maximal slope of the aggregation curve was automatically calculated from the tangents at the steepest slope of the aggregation curve and expressed in Ω/min. The time period required for the onset of aggregation after collagen addition (lag time) was automatically measured and expressed in seconds.

DATA ANALYSIS

Assay reproducibility was assessed by calculation of the coefficient of variation of duplicate aggregation measurements. All subsequent analyses were based on the first data point of each duplicate measurement set, which should fall within the predefined acceptable assay limit of 15%. Intra-individual variability in platelet aggregation (amplitude) was assessed on Day 1 (no ASA) and on Day 7 (on ASA-treatment). For assessment of ASA effects on platelet aggregation, the average maximal aggregation amplitude was calculated for each collagen concentration based on the first time point on Day 1 (pre-ASA) and

the first time point of Day 7 (post-ASA, before final ASA administration). All data are presented as mean ± standard deviation unless indicated otherwise. A power calculation was performed to estimate the desired sample sizes for future ASA-drug interaction studies with a cross-over design, based on the observed intra-individual variability in aggregation amplitude on Day 7. A log-normal data distribution was assumed, and the calculation was performed at 90% nominal power to demonstrate a treatment effect of 50% and two-sided significance level of 5%.

Results

INDUCTION OF WHOLE BLOOD PLATELET AGGREGATION BY COLLAGEN

The relationship between collagen concentration and platelet aggregation response (defined by aggregation amplitude) was assessed in fresh whole blood samples from 6 healthy volunteers (Figure 1). A maximal aggregation response of approximately 15–16 Ω was reached at 1.0 and 2.0 µg/mL collagen (Figure 1). Assay reproducibility was adequate for all collagen concentrations tested, with a coefficient of variation of duplicate measurements within the predefined acceptable assay limit of 15% (data not shown). Over the 6.5 hours observation period on Day 1, intra-subject variability was dependent on the applied collagen concentration, with the lower observed variability at higher collagen concentrations. The intra-subject coefficient of variation (CV) was 22.7% at 0.5 µg/mL collagen and 9.9% at 2.0 µg/mL collagen. The response in aggregation rate mirrored the response in aggregation amplitude while increasing collagen concentrations decreased the aggregation lag time (data not shown).

INHIBITION OF COLLAGEN-INDUCED PLATELET AGGREGATION BY ASA

Daily administration of 75 mg ASA for 5 consecutive days strongly reduced maximal platelet aggregation, as expressed in amplitude, for collagen concentrations between 0.5 and 2.0 µg/mL. At low collagen concentrations, platelet aggregation was almost completely inhibited by ASA treatment (Figure 1, reduction from 9.0 ± 5.3 Ω to 1.8 ± 0.4 Ω at 0.5 µg/mL collagen). At higher collagen concentrations, the ASA-induced reduction in platelet aggregation was less pronounced (Figure 1, from 15.3 ± 4.6 Ω to 4.2 ± 0.8 Ω at 1.0 µg/mL collagen, and from 15.5 ± 2.4 Ω to 9.7 ± 4.6 Ω at 2.0 µg/mL collagen).

The effect of ASA therapy on collagen-induced platelet aggregation was also assessed by aggregation rate and lag time. Aggregation rate showed an ASA effect pattern that was comparable with the pattern observed for aggregation amplitude and lag time followed a similar, but reverse pattern (data not shown).

Any additional effect of the last ASA administration on Day 7 on platelet aggregation was not observed: ASA administration for 5 consecutive days had already resulted in a maximal inhibition of aggregation for all parameters tested (data not shown). The observed intra-subject variability for platelet aggregation amplitude on ASA treatment (assessed over the course of Day 7) was 36.7% and 32.5% for 1 and 2 µg/mL collagen, respectively (Table 1). Based on these variabilities, power calculations were performed demonstrating that group sizes of 11 and 9 subjects (for 1 and 2 µg/mL collagen, respectively) would be required to demonstrate a treatment effect of 50% in future ASA-drug interaction studies with a cross-over design (Table 1).

Discussion

Routine low dose ASA therapy is frequently applied as cardiovascular disease prophylaxis.[14] This therapy is mostly long-term and concomitant medication use is almost inevitable.[3] Hence a methodology to evaluate potential interactions affecting platelet function between ASA and co-medication is desired. We developed and characterized a platelet aggregometry methodology for assessment of potential ASA-drug interactions affecting platelet aggregation. We chose an experimental setup that optimally represents human physiology (whole blood as matrix, collagen as inducer), and demonstrated method robustness (in terms of assay reproducibility and intra-subject variability). As such, the desired criteria for methodology evaluating potential interactions between ASA and any other drug in a clinical study were met.

A range of collagen concentrations was selected to induce platelet aggregation in fresh whole blood samples including a concentration of 1.0 µg/mL which is often used in the clinical assessment of ASA effects.[15,16] A clear relationship between collagen concentration and aggregation amplitude response was observed, with an aggregation level of 9 Ω at 0.5 µg/mL and a maximal aggregation of 15–16 Ω at 1.0 and 2.0 µg/mL. Inter-individual differences in responsiveness to collagen were observed, with higher variability at

lower collagen concentrations. This can be attributed to the fact that 2 subjects showed a low aggregation at 0.5 µg/mL collagen, whereas the other 4 subjects had already (sub-)maximal aggregation at this collagen concentration. These results suggest the existence of an individual collagen threshold concentration that triggers platelet aggregation to exceed a certain basal level (approximately 1–5 Ω) via an all-or-nothing response; when the collagen stimulus is strong enough, a platelet aggregation response is evoked and augmented through the strong self-amplifying loops of the haemostatic cascade. This is consistent with Kawasaki *et al.* who described comparable differences in platelet sensitivity to collagen in PRP,[17] and with our earlier findings.[6]

We demonstrated that platelet aggregation was effectively inhibited after 6 days of daily 75 mg ASA treatment, which is in line with other studies.[7,18] The degree of inhibition by ASA treatment was determined by the collagen concentration and ranged from 80% to 38% (for 0.5 and 2.0 µg/mL collagen respectively). We also assessed the effect of ASA treatment on platelet aggregation by slope (aggregation rate) and lag time (aggregation onset), but found that for assessment of ASA effects all three readout parameters were generally interchangeable. This is consistent with the mode of action of ASA. Since amplitude is the most frequently used readout parameter of platelet aggregation, we considered this parameter to be the preferred parameter for the future ASA-drug interaction studies. The desired level of platelet aggregation, assessed by amplitude, for ASA-drug interaction studies should allow demonstration of both drug-induced reductions and elevations on an ASA background. Since lower collagen concentrations of 0.5 and 0.75 µg/mL result in minimal platelet aggregation levels in ASA-treated volunteers, these concentrations are not suitable from a methodological perspective. In contrast, higher collagen concentrations (1 to 2 µg/mL) do offer a sufficient window for demonstration of potential drug-induced changes in platelet aggregation. The observed intra-subject variability in aggregation amplitude at 1 and 2 µg/mL collagen on Day 7 was used to assess the required sample size for future ASA-drug interaction studies with a cross-over design. A sample size of 11 and 9 subjects would provide sufficient power to detect a 50% treatment effect at 1 and 2 µg/mL collagen, respectively. A recent ASA-sugammadex interaction study that applied the described methodology that we describe here, confirms the reported intra-subject variability and estimated sample size.[19] The observed intra-subject CV in this study was 20–23%, with an actual sample size of 23 subjects, at a collagen concentration of 1.5 µg/mL. This variability was sufficiently low to confirm the absence of an ASA-sugammadex interaction, defined as a 33% further inhibition in platelet aggregation. This demonstrates that the findings in our relatively small sample size of 6 subjects can be translated to larger sample sizes, and that our collagen-induced whole blood aggregation methodology is feasible for evaluation of ASA-drug interactions.

Conclusions

Overall, our experiments support the feasibility of collagen-induced whole blood aggregation to evaluate ASA-drug interactions in healthy male volunteers. The methodology that we developed optimally represents human physiology, and demonstrated method robustness in terms of assay reproducibility and intra-subject variability. Based on our experiments and subsequent power calculations, a collagen concentration of 1–2 µg/mL and a minimal group size of 9–11 subjects should be selected for future cross-over ASA-drug interaction studies assessing platelet aggregation effects of a drug-of-choice. This setup will result in a platelet aggregation level (expressed in aggregation amplitude) that offers sufficient window for demonstration of drug-induced changes.

REFERENCES

1 S.F. de Meyer, K. Vanhoorelbeke, K. Broos, Salles, I.I., *et al., Antiplatelet drugs.* Br J Haematol, 2008. 142(4): p. 515-528.

2 G.H. Guyatt, E.A. Akl, M. Crowther, D.D. Gutterman, *et al., Executive summary: Antithrombotic Therapy and Prevention of Thrombosis, 9th ed: American College of Chest Physicians Evidence-Based Clinical Practice Guidelines.* Chest, 2012. 141(2 Suppl): p. 7S-47S.

3 G. Feher, K. Koltai, E. Papp, B. Alkonyi, *et al., Aspirin resistance: possible roles of cardiovascular risk factors, previous disease history, concomitant medications and haemorrheological variables.* Drugs Aging, 2006. 23(7): p. 559-567.

4 D.C. Cardinal and R.J. Flower, *The electronic aggregometer: a novel device for assessing platelet behavior in blood.* J Pharmacol Methods, 1980. 3(2): p. 135-158.

5 A.M. Dyszkiewicz-Korpanty, E.P. Frenkel, and R. Sarode, *Approach to the assessment of platelet function: comparison between optical-based platelet-rich plasma and impedance-based whole blood platelet aggregation methods.* Clin Appl Thromb Hemost, 2005. 11(1): p. 25-35.

6 M. Moerland, M.J. Kemme, M. van der Linden, and J. Burggraaf, *Measurement of collagen- and serotonin-induced platelet aggregation in whole blood.* Expert Rev Clin Pharmacol, 2010. 3(2): p. 177-182.

7 J.P. de la Cruz, S. Camara, I. Bellido, T. Carrasco, *et al., Platelet aggregation in human whole blood after chronic administration of aspirin.* Thromb Res, 1987. 46(1): p. 133-140.

8 B.T. Ivandic, E. Giannitsis, P. Schlick, P. Staritz, *et al., Determination of aspirin responsiveness by use of whole blood platelet aggregometry.* Clin Chem, 2007. 53(4): p. 614-619.

9 D.L. McGlasson and G.A. Fritsma, *Comparison of four laboratory methods to assess aspirin sensitivity.* Blood Coagul Fibrinolysis, 2008. 19(2): p. 120-123.

10 S.H. Meves, H. Neubauer, U. Overbeck, and H.G. Endres, *Is there an ideal way to initiate antiplatelet therapy with aspirin? A crossover study on healthy volunteers evaluating different dosing schemes with whole blood aggregometry.* BMC Res Notes, 2011. 4(106): p. 1-8.

11 H. Neubauer, A.F. Kaiser, H.G. Endres, J.C. Kruger, *et al., Tailored antiplatelet therapy can overcome clopidogrel and aspirin resistance - the BOchum CLopidogrel and Aspirin Plan (BOCLA-Plan) to improve antiplatelet therapy.* BMC Med, 2011. 9(3): p. 1-12.

12 G. Spectre, L. Arnetz, C.G. Ostenson, K. Brismar, *et al., Twice daily dosing of aspirin improves platelet inhibition in whole blood in patients with type 2 diabetes mellitus and micro- or macrovascular complications.* Thromb Haemost, 2011. 106(3): p. 491-499.

13 B. Nieswandt and S.P. Watson, *Platelet-collagen interaction: is GPVI the central receptor?* Blood, 2003. 102(2): p. 449-461.

14 C.L. Campbell, S. Smyth, G. Montalescot, and S.R. Steinhubl, *Aspirin dose for the prevention of cardiovascular disease: a systematic review.* JAMA, 2007. 297(18): p. 2018-2024.

15 D.L. McGlasson and G.A. Fritsma, *Whole blood platelet aggregometry and platelet function testing.* Semin Thromb Hemost, 2009. 35(2): p. 168-180.

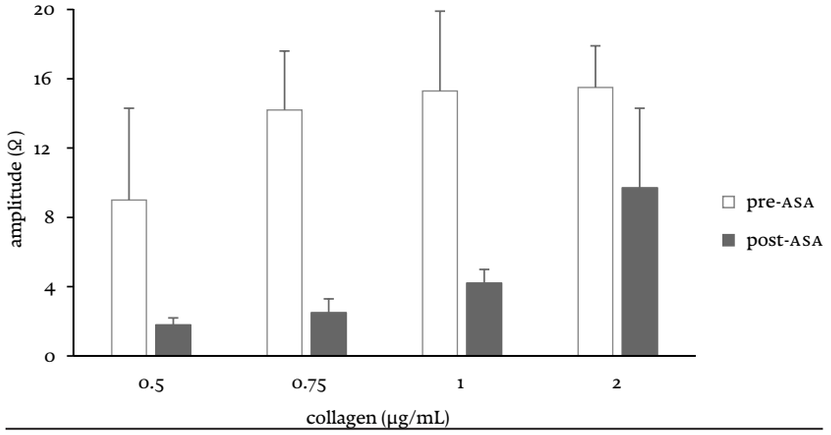
16 H.K. Breddin, *Can platelet aggregometry be standardized?* Platelets, 2005. 16(3-4): p. 151-158.

17 T. Kawasaki, Y. Ozeki, T. Igawa, and J. Kambayashi, *Increased platelet sensitivity to collagen in individuals resistant to low-dose aspirin.* Stroke, 2000. 31(3): p. 591-595.

18 H. Kariyazono, K. Nakamura, J. Arima, O. Ayukawa, *et al., Evaluation of antiplatelet aggregatory effects of aspirin, cilostazol and ramatroban on platelet-rich plasma and whole blood.* Blood Coagul Fibrinolysis, 2004. 15(2): p. 157-167.

19 P.J. de Kam, R. El Galta, A.C. Kruithof, H. Fennema, *et al., No clinically relevant interaction between sugammadex and aspirin on platelet aggregation and coagulation parameters.* Int J Clin Pharmacol Ther, 2013. 51(12): p. 976-985.

Figure 1 Mean maximal aggregation expressed as amplitude of impedance (Ω) before ASA administration (pre-ASA) and after 75 mg ASA administration once daily for 5 consecutive days (post-ASA) in response to varying collagen concentrations. Error bars represent standard deviation, n = 6.



ASA = aspirin administration.

Table 1 Power calculation for ASA-drug interaction studies with a cross-over design assessing amplitude of platelet aggregation at 1 or 2 µg/mL collagen, based on the intra-subject variability post-ASA (n = 6) assuming a log-normal distribution, at 90% nominal power to demonstrate a treatment effect of 50% and two-sided significance level of 5%.

Collagen concentration (µg/mL)	Intra-subject CV post-ASA (%)	Actual power (%)	Sample size
1.0	36.7	91.8	11
2.0	32.5	92.5	9

ASA = aspirin; and CV = coefficient of variation.

CHAPTER 5

No clinically relevant interaction between sugammadex and aspirin on platelet aggregation and coagulation parameters

International Journal of Clinical Pharmacology and Therapeutics, 2013. 51(12): p. 976-985, doi: 10.5414/CP201970

Pieter-Jan de Kam, Rachid El Galta, Annelieke C. Kruithof, Hein Fennema, Marie-José van Lierop, Katsuhiro Mihara, Jacobus Burggraaf, Matthijs Moerland, Pierre Peeters, and Matthew D. Troyer

Abstract

OBJECTIVES This study evaluated interaction potential between sugammadex and aspirin on platelet aggregation.

METHODS This was a randomized, double-blind, placebo-controlled, four-period crossover study in 26 healthy adult males. Treatments were intravenous placebo, intravenous sugammadex 4 mg/kg, and intravenous placebo/sugammadex with once-daily oral aspirin 75 mg. Primary objective was to assess interaction between sugammadex and aspirin on platelet aggregation using collagen-induced whole-blood aggregometry. Effects on activated partial thromboplastin time (APTT) and cutaneous bleeding time were also evaluated. Platelet aggregation and APTT were evaluated by geometric mean ratios, using area-under-effect curves 3–30 minutes after sugammadex/placebo dosing. Bleeding time ratio was evaluated at 5 minutes post-dosing. Non-inferiority margins were pre-specified via literature review. Type I error was controlled using a hierarchical strategy.

RESULTS Ratio for platelet aggregation for aspirin with sugammadex *versus* aspirin alone was 1.01, with lower limit of two-sided 90% confidence interval (CI) of 0.91 (above non-inferiority margin of 0.75). Ratio for statistical interaction between sugammadex and aspirin on APTT was 1.01, with upper 90% CI of 1.04 (below non-inferiority margin of 1.50), and for sugammadex *versus* placebo alone was 1.06, with an upper 90% CI of 1.07 (below non-inferiority margin of 1.50). Ratio for bleeding time for aspirin with sugammadex *versus* aspirin plus placebo was 1.20, with upper 90% CI of 1.45 (below non-inferiority margin of 1.50). Sugammadex was generally well tolerated.

CONCLUSION There was no clinically relevant reduction in platelet aggregation with addition of sugammadex 4 mg/kg to aspirin. Pre-determined non-inferiority margins were not exceeded for bleeding time and APTT.

Introduction

The selective muscle relaxant-binding agent sugammadex (Bridion®, Merck Sharp & Dohme Corp., Oss, The Netherlands) is a modified γ-cyclodextrin which encapsulates the steroidal neuromuscular blocking agents rocuronium and vecuronium, thereby reversing neuromuscular blockade (NMB).[1,2] Sugammadex provides rapid and complete NMB reversal, with a good overall safety profile[3–6] and is not associated with an increased risk of post-surgical bleeding or blood loss, compared to standard care.[7] Sugammadex has been approved for reversal of rocuronium- and vecuronium-induced moderate and deep NMB in more than 70 countries.

Initial pre-clinical *in vitro* spiking experiments with relatively high concentrations of sugammadex indicated prolongation of activated partial thromboplastin time (APTT) and prothrombin time (PT), with a subsequent exploratory study suggesting a dose-related effect with limited (≤ 20%) and transient (≤ 30 minutes) prolongations of APTT and PT(international normalized ratio (INR)) after the highest dose of sugammadex (16 mg/kg) (Merck, data on file). Additional *in vitro* studies suggested that these limited and transient effects on APTT and PT(INR) after sugammadex could be explained by a sugammadex-induced decrease in formation and activity of factor Xa (Merck, data on file).

In clinical practice, aspirin (acetylsalicylic acid) is often used for primary and secondary prevention of arterial disease. Indeed, in a recent study, almost 20% of the entire adult population of the United States of America (USA) reported taking aspirin every day or every other day; with this incidence rising to almost 50% in those aged > 65 years.[8] Furthermore, in patients with cardiovascular disease and diabetes, aspirin intake has been shown to be ≈83% and ≈63%, respectively.[9] Aspirin exerts an antiplatelet effect by inhibiting the cyclooxygenase-1 (COX-1) enzyme and subsequent production of thromboxane A_2. While the mechanism of action of aspirin and sugammadex are different, with previous *in vitro* tests indicating no effect of sugammadex on platelet function, it was still considered important to perform a dedicated *in vivo* interaction study, with sugammadex and aspirin on platelet aggregation. Platelet aggregation, measured by whole blood aggregometry in response to collagen, was chosen as the primary endpoint, as it has been shown to be a reliable and relevant parameter for antiplatelet agents such as aspirin.[10] Secondary parameters consisted of the APTT and cutaneous bleeding time.

There is widespread consensus that aspirin 75–100 mg is an acceptable cardioprotective dose range.[11] For the present study, a dosage of 75 mg/day was selected as the minimum cardioprotective dose level which would provide a level of platelet aggregation inhibition that would allow a sufficient window to evaluate a potential interaction between sugammadex and aspirin.[12,13]

Methods

STUDY DESIGN

This was a single-center, placebo-controlled, four-period crossover study in healthy volunteers performed at the Centre for Human Drug Research (CHDR), Leiden, The Netherlands. The study was double-blind for sugammadex *versus* placebo, with open-label administration of aspirin. The study was conducted in accordance with principles of Good Clinical Practice and was approved by the Medical Ethics Committee of Leiden University Medical Center (The Netherlands). Subjects included in the study were required to provide written, informed consent.

SUBJECTS

The study participants were healthy male volunteers of any ethnicity. To be eligible for inclusion, subjects were required to be 18–45 years of age and have a body mass index of 18–32 kg/m^2. Clinical laboratory tests had to be within normal or clinically acceptable limits, including normal liver function test results at screening (within 3 weeks of study drug administration) and on the day before dosing (Day –1), and electrocardiogram (ECG) and vital signs within normal ranges. Values for platelet aggregation, APTT and PT at screening, and APTT and PT on Day –1, had to be within normal limits. Screening and predose bleeding time had to be within 4 minutes, and platelet count at screening had to be within the range 150,000–380,000/μL.

Subjects were excluded from the trial if they had a history of sensitivity to chemically related compounds or excipients that could be employed in the study, or any surgical or medical condition that could possibly influence the pharmacokinetics and/or dynamics of any drug used. Subjects were also excluded if they were unable to refrain from all use of xanthine-containing food products and alcohol from 48 hours prior to dosing of trial medication, and from all use of products containing grapefruit from 7 days prior to dosing of trial medication, until the last blood sample was taken. Other exclusion criteria included previous participation in another clinical trial within the preceding 3 months, history of alcohol or drug abuse, smoking more than 10 cigarettes per day, blood donation within the past 60 days, and any other relevant medical history (*e.g.* gastrointestinal bleeding, easy bruising, or frequent nose bleeds).

Failure to comply with the requirements of the study, including subjects with serum thromboxane B_2 levels below 50 ng/mL at Day –1 assessment in Period 2, resulted in discontinuation from the study.

ENDPOINTS

The primary endpoint was platelet aggregation, with the primary comparison being sugammadex plus aspirin *versus* aspirin alone. Secondary endpoints included APTT (comparison between sugammadex and aspirin, and comparison of sugammadex alone *versus* placebo), and bleeding time (sugammadex plus aspirin *versus* aspirin alone). Exploratory endpoints included PT corrected INR.

Tolerability and safety were also evaluated, including reporting of adverse events (AEs) and serious AEs, coded according to the Medical Dictionary of Regulatory Activities (MedDRA) version 14.0, vital signs, clinical laboratory safety parameters, ECG, and physical examination. Primary and secondary endpoints and safety were reported for all patients who received treatment according to the randomization schedule (all-subjects-treated group).

Pharmacokinetic evaluations were performed in all patients who received sugammadex in two study periods, and included maximum plasma concentration (C_{max}) and area under the concentration-time curve from 0 to 6 hours ($AUC_{0-6\ h}$) for sugammadex alone and sugammadex plus aspirin.

PROCEDURES

Subjects were screened for eligibility within 3 weeks prior to study drug infusion and were randomized to one of four treatment sequences (Figure 1). In the morning of Day 1 of each treatment period, subjects received a single intravenous dose of sugammadex 4 mg/kg or placebo (NaCl 0.9%) in the absence (Periods 1 and 2) or presence (Periods 3 and 4) of aspirin. There was a washout of at least 4 days between each treatment period. Sugammadex or placebo (equal volume) was administered in a forearm vein as a 10-second injection. For at least 7 days prior to Period 3, until the day of sugammadex or placebo administration in Period 4 (maximum 16 days), subjects were treated at the study site with once-daily oral aspirin 75 mg (Actavis®) at

approximately the same time of day as the administration of sugammadex or placebo in the previous periods, with sugammadex or placebo given ≈30 minutes after aspirin dosing in Periods 3 and 4. Due to the risk of gastrointestinal problems if aspirin is taken on an empty stomach, a meal was provided on each treatment day of sugammadex or placebo, ≈0.5–1.0 hours before aspirin intake (*i.e.* 1.0–1.5 hours before sugammadex or placebo administration).

Blood samples for assessment of sugammadex concentrations, platelet aggregation, APTT, PT and activated clotting time (ACT) were collected from the arm opposite to that used for drug infusion 16 minutes before the sugammadex or placebo dose, and then at 3, 15, and 30 minutes, and 1, 3, and 6 hours post-dose. Cutaneous bleeding time, defined as the time at which bleeding stops following the infliction of a standard incision, was assessed pre-dose (–15 minutes), at 5 minutes and 6 hours post-dose. The bleeding time assessment was performed in the same arm as was used for study drug infusion using the Ivy bleeding time technique.[14] In Period 1, extra blood samples for pharmacogenetic analysis were drawn. In addition, blood samples for thromboxane B_2 assay were obtained on Day –1 in Periods 1 and 2, as an extra check to determine whether the subject was free of use of antiplatelet drugs.

Platelet aggregation was measured immediately after blood collection using ≈5 mL of whole blood collected in a citrated tube. Platelet aggregation was induced with 1.5 μg/mL collagen, and assessed using a validated whole blood impedance aggregometry (Chrono-log model 590, Chrono-log Corporation)[10], with results expressed as ohms of impedance.

APTT and PT were measured at Good Biomarker Sciences (Leiden, The Netherlands) using standard procedure: TriniClot Automated APTT, TriniClot PT HTF, Accuclot 1 and 2 for quality control (Trinity Biotech, Kordia Lifesciences, Leiden), and an automated clotting analyzer (AMAX Destiny Plus, Kordia Lifesciences, Leiden, The Netherlands). ACT was assessed using the Hemochron® Response whole blood coagulation system with P214 tubes according to the manufacturer's instructions. Sugammadex plasma concentrations were determined using a validated liquid chromatographic assay with mass spectrometric detection.[15] Samples taken after the placebo treatment were not analyzed.

HYPOTHESES

The primary hypothesis was that administration of a single dose of sugammadex 4 mg/kg following multiple doses of aspirin 75 mg would not cause inhibition of collagen-induced platelet aggregation additional to that caused by multiple dose aspirin alone. To assess platelet aggregation, the area under the effect curve between 3 to 30 minutes after drug administration divided by time span ($AUEC_{3-30\,min}$) was calculated. A geometric mean ratio (GMR) in $AUEC_{3-30\,min}$ of sugammadex with aspirin *versus* aspirin alone < 0.75 was considered a clinically meaningful effect.

Secondary hypotheses were:

- Administration of single dose sugammadex 4 mg/kg following multiple doses of aspirin 75 mg would not increase APTT to a greater extent than the multiplicative effect of sugammadex and aspirin alone. *i.e.* the true ratio of $AUEC_{3-30\,min}$ GMR of sugammadex with aspirin *versus* aspirin alone to $AUEC_{3-30\,min}$ GMR of sugammadex alone *versus* placebo > 1.5 would be considered a clinically meaningful effect.
- Administration of single dose sugammadex 4 mg/kg would not increase APTT to a greater extent than placebo. A GMR in $AUEC_{3-30\,min}$ of sugammadex *versus* placebo > 1.5 would be considered a clinically meaningful effect.
- Administration of single dose sugammadex 4 mg/kg following multiple doses of aspirin 75 mg would not have a clinically meaningful additional effect on bleeding time after 5 minutes, compared with multiple dose aspirin alone. That is, the true GMR of sugammadex with aspirin *versus* aspirin alone would not exceed 1.5.

SAMPLE SIZE

An initial sample size of 24 subjects was planned, as this was calculated to have at least 90% power to reject the primary null hypothesis ($AUEC_{3-30\,min}$ GMR < 0.75) at a one-sided significance level of 5%, assuming a true GMR in platelet aggregation $AUEC_{3-30\,min}$ of sugammadex and aspirin combination to aspirin alone of 1, and a within-subject coefficient of variation (CV) of 35%. A blinded evaluation of the within-subject CV after aspirin treatment was planned as follows, in order to evaluate the sample size after 23 subjects had completed the study: if the estimated within-subject CV was (i) ≤ 0.35, the study was to be considered complete, proceeding to final analysis; (ii) > 0.35 and ≤ 0.40, the sample size was to be re-estimated based on the above sample size assumptions; (iii) > 0.4 and ≤ 0.50, a maximum of 36 randomized subjects were to be used; and (iv) > 0.50, the study was to be terminated once the 24 enrolled subjects completed the study. The results of the blinded sample size reevaluation showed an estimated within-subject < 0.35 and confirmed that the planned sample size was sufficient to test the primary hypothesis with at least 90% power, at a one-sided significance level of 5%.

Baseline characteristics were summarized using descriptive statistics by treatment group. Mean and standard deviation were reported for continuous variables and number and percentage of subjects were reported for categorical variables.

STATISTICAL ANALYSES

The primary parameter for assessing the primary endpoint platelet aggregation and the secondary endpoint APTT was $AUEC_{3-30\ min}$ after dosing of the study drug, divided by the time span. The potential for interaction between sugammadex and aspirin on platelet aggregation was evaluated by $AUEC_{3-30\ min}$ GMR, with relevant limit of the corresponding two-sided 90% confidence interval (CI).

The analyses were carried out using a mixed model for repeated measures with the log transformed $AUEC_{3-30\ min}$ as a dependent variable, treatment and sequence as factors, and log baseline platelet aggregation as a covariate. The potential for interaction between sugammadex and aspirin on APTT was evaluated similarly to platelet aggregation.

For bleeding time, the potential for interaction between sugammadex and aspirin was evaluated by the GMR at 5 minutes after dosing based on a mixed model for repeated measures with the log transformed bleeding time as a dependent variable, treatment and sequence as factors, and log baseline bleeding time as a covariate.

For the primary endpoint (platelet aggregation), if the lower limit of the two-sided 90% CI of the GMR of sugammadex in combination with aspirin *versus* aspirin alone was above 0.75, non-inferiority would be declared. The bound of 0.75 for platelet aggregation was determined based on the results of clinical studies assessing effects on whole blood platelet aggregation after administration of low-dose clopidogrel (75 mg) on top of aspirin.[12,16,17] For the secondary endpoints APTT and bleeding time, a conclusion of non-inferiority would be drawn if the upper limit of the two-sided 90% CI of the GMR for the relevant comparison was below 1.5. A Type I error of 5% was controlled by adjustment for multiplicity using a hierarchical strategy starting with the primary comparison of sugammadex plus aspirin *versus* aspirin alone on platelet aggregation, followed by testing sugammadex by aspirin statistical interaction on APTT, then the comparison of sugammadex alone *versus* placebo on APTT and lastly the comparison of sugammadex plus aspirin *versus* aspirin alone on bleeding time.

Safety analyses were performed for the all-subjects-treated (AST) group (all randomized subjects who received at least 1 dose of study medication). Pharmacodynamic assessments were performed for the all-subjects-evaluable group, which consisted of all subjects from the AST group for whom at least one pharmacodynamic parameter could be calculated. Pharmacokinetic assessments were performed for the all-subjects-pharmacokinetically-evaluable group (all treated subjects with at least 1 post-dosing pharmacokinetic sample. For pharmacokinetic parameters (C_{max} and $AUC_{0-6\,h}$), descriptive statistics only were reported.

Results

SUBJECTS

Of 24 patients initially randomized and treated, 2 were prematurely discontinued, 1 because of poor venous access for repeated blood sampling and 1 because of a mild hypersensitivity reaction after sugammadex given in Period 1. These 2 subjects were replaced by 2 subjects who followed the same randomization treatment sequence. The total number of 26 treated subjects all received treatment according to their randomization schedule. Hence the AST group comprised 26 subjects. One of the replacement subjects was discontinued after completion of two study periods because of a thromboxane B_2 level < 50 ng/mL prior to the second dosing. Thus, 23 subjects completed the study.

In total, there were 20 Caucasian and 6 non-Caucasian male subjects included in the study. Mean (standard deviation (SD)) age was 25.7 ±7.4 years, with a mean (SD) body mass index (BMI) of 22.8 ±2.9 kg/m^2 (weight 76.5 ±9.3 kg and height 183.4 ± 8.0 cm).

PRIMARY AND SECONDARY ENDPOINTS

Unadjusted GMRs (with corresponding two-sided 90% CI) for the combination of sugammadex plus aspirin *versus* aspirin alone, and of sugammadex *versus* placebo, are shown in Figure 2 for platelet aggregation, APTT and bleeding time. The results of the primary and secondary endpoint analyses are shown in Table 1. The GMR for platelet aggregation for aspirin in combination with sugammadex *versus* aspirin alone was 1.01, with a corresponding lower limit of the 90% CI of 0.91; thus above the non-inferiority margin of 0.75. Moreover, the GMR for sugammadex alone *versus* placebo alone

was 0.99, with a corresponding lower limit of the 90% CI of 0.96 (Table 1). The GMR of the statistical interaction between sugammadex and aspirin on platelet aggregation (*i.e.*, the ratio of GMR for aspirin in combination with sugammadex *versus* aspirin alone to the GMR of sugammadex alone *versus* placebo alone) was 1.02, with a corresponding lower limit of the 90% CI of 0.91 (Table 1).

In addition, none of the statistical interactions and/or effects on APTT exceeded the pre-specified non-inferiority margin of clinical relevance (Table 1). Similarly, any effects of sugammadex on bleeding time did not exceed the pre-specified non-inferiority margin (Table 1); GMR of aspirin in combination with sugammadex *versus* aspirin alone was 1.2 (corresponding upper limit of the 90% CI of 1.45) and for the statistical interaction between sugammadex and aspirin was 1.12 (corresponding upper limit of the 90% CI of 1.44) (Table 1). However, the effects of aspirin in combination with sugammadex *versus* aspirin alone on bleeding time showed considerable variability, and were not statistically significant. Moreover, the unadjusted bleeding time GMR for baseline of aspirin in combination with sugammadex *versus* aspirin alone at 5 minutes shown was ≈1.1 (Figure 2C).

EXPLORATORY ENDPOINTS

Sugammadex-only dosing resulted in a small increase in PT(INR) $AUEC_{3\text{–}30\ min}$ of 5% *versus* placebo; this increase was similar (4%) for sugammadex in combination with aspirin *versus* aspirin alone.

The GMR for ACT $AUEC_{3\text{–}30\ min}$ was similar for aspirin in combination with sugammadex *versus* sugammadex alone (1.0), and sugammadex alone *versus* placebo (0.99).

MEAN VALUES FOR THE KEY PARAMETERS OVER TIME

Platelet aggregation was on average 17 ohms in the treatment arms that did not receive aspirin, and was relatively stable over the day (Figure 3A). Aspirin therapy inhibited baseline collagen-induced platelet aggregation (before placebo administration) by ≈25% (average platelet aggregation 13 ohms).

The secondary parameter APTT was on average 32–33 seconds in the placebo treatment arm for all time points and was not affected by aspirin therapy (Figure 3B). Sugammadex dosing induced a limited prolongation, relative to pre-dose, of ≈3.5 seconds for the sugammadex only group, resulting in a mean (SD) APTT of 35.9 (3.6) seconds, and a prolongation of ≈7 seconds for the aspirin and sugammadex combination group, resulting in a mean (SD) APTT of 40.2 (16.7) seconds. In both cases there was a return to baseline levels after ≈30 minutes post-dose (Figure 3B).

Bleeding time showed high variability (≈10 seconds) over the observation period after placebo (Figure 3C). Sugammadex treatment did not affect this bleeding time pattern. Aspirin prolonged the bleeding time to 134 seconds at pre-dose in the treatment arm with placebo and to 116 seconds in the treatment arm with sugammadex (Figure 3C).

PT(INR) showed an increase of ≈10% at 3 minutes post-dose for both sugammadex alone *versus* placebo and sugammadex in combination with aspirin *versus* aspirin alone. PT(INR) returned towards baseline levels after ≈30 minutes post-dose.

SAFETY

Sugammadex, either alone or in combination with aspirin, was generally well tolerated by all 26 treated subjects. One subject reported dyspnea and urticaria of mild intensity immediately after sugammadex administration. The investigator considered that this subject experienced a mild hypersensitivity reaction and decided to discontinue this subject from the study. All adverse events observed in this study were of mild intensity and no clinically relevant changes in laboratory parameters, vital signs or ECG were observed.

PHARMACOKINETICS

Twenty-three subjects received sugammadex in two periods and were evaluable for pharmacokinetic assessment. Mean C_{max} and $AUC_{0\text{–}6\ h}$ were comparable for sugammadex alone and sugammadex plus aspirin (Table 2).

Discussion

This study was performed to investigate the potential interaction between sugammadex 4 mg/kg and aspirin on platelet aggregation using collagen-induced whole blood aggregometry in healthy male volunteers. The sugammadex 4 mg/kg dose was selected as this is the highest dose recommended for routine NMB reversal, and is frequently used in clinical practice.

The results of this study showed no clinically relevant reduction in platelet aggregation with the addition of sugammadex 4 mg/kg to background aspirin therapy. The GMR for platelet aggregation for aspirin in combination with

sugammadex *versus* aspirin alone was 1.01. The corresponding lower limit of the 90% CI was 0.91; well above the pre-defined non-inferiority margin of 0.75. Combined, these results indicate that there was no clinically relevant effect of sugammadex on platelet aggregation when combined with aspirin.

It was chosen to measure platelet aggregation by whole blood aggregometry in response to collagen, using an impedance aggregometer. This method of evaluation was considered preferable over turbidometric platelet aggregation in platelet rich plasma as it mimics more closely the physiological situation,[18] and utilizes collagen, a highly potent platelet activator, as an inducer. Results of a preliminary pilot study, which measured platelet aggregation induced by a collagen concentration range of 0.5–2.0 µg/mL, indicated that 75 mg aspirin treatment in the setting of a collagen concentration of 1.5 µg/mL would be expected to result in a level of inhibition of whole blood platelet aggregation that would provide an acceptable level of variability and sufficient margin to evaluate any potential interaction of sugammadex in combination with aspirin on platelet aggregation. Acceptable variability was confirmed during the planned blinded evaluation of variability in the present study, which occurred after 23 subjects had completed the study.

It was important to also assess the potential interaction between sugammadex and aspirin on APTT, as *in vitro* and *in vivo* results indicated a limited (≈10%) and transient (≤ 30 minutes) prolongation of APTT after 4 mg/kg sugammadex administration. In this study the GMR of the statistical interaction between sugammadex and aspirin on APTT was 1.01, with an upper limit of the 90% CI of 1.04 (well below the pre-specified non-inferiority margin of 1.5). Furthermore, for sugammadex alone *versus* placebo alone, the GMR was 1.06, with an upper limit of the 90% CI of 1.07 (again well below the pre-specified non-inferiority margin). The observed mean increase in APTT GMR increase of 6% during the 30 minutes after sugammadex administration was statistically significant, but the magnitude was not considered clinically relevant.

Recent randomized, double-blind studies have also demonstrated the absence of clinically relevant interaction effects of sugammadex on anti-Xa or APTT, when added to background enoxaparin or unfractionated heparin; and on bleeding events in surgical patients receiving anticoagulants. These results further support the lack of interaction effects of sugammadex with agents used for anticoagulation (Merck, data on file).[7,19]

As a marker for coagulation, bleeding time is known to exhibit high variability,[20–22] and this was also the case in this study. Nevertheless, bleeding time was evaluated, to further strengthening the observations on platelet function as assessed with whole blood platelet aggregometry. There was an estimated 20% increase in bleeding time 5 minutes after sugammadex plus aspirin compared with aspirin alone; however, this (as well as the upper limit of the two-sided 90% CI) was below the pre-defined non-inferiority margin of 50% and was not statistically significant.

Sugammadex pharmacokinetic parameters were similar following sugammadex both alone and with aspirin, and were consistent with previous pharmacokinetic studies, confirming no pharmacokinetic drug-drug interaction between sugammadex and aspirin.

Sugammadex, either alone or in combination with aspirin, was generally well tolerated in the healthy young male volunteers in this study. Only 1 subject discontinued the study due to an AE, following what appeared to be a mild hypersensitivity reaction to sugammadex. Importantly, all AEs observed during the study were mild, and were generally similar across treatment groups.

Conclusions

There was no reduction in platelet aggregation with the addition of sugammadex 4 mg/kg to background aspirin therapy at a clinically relevant prophylactic dose (75 mg) in healthy young males, and any clinically meaningful reduction could be confidently excluded. In addition, for the secondary parameters APTT and bleeding time, the pre-specified non-inferiority margins of clinical relevance were not exceeded after the addition of sugammadex 4 mg/kg to background aspirin therapy. Sugammadex, either alone or in combination with aspirin, was generally well tolerated in this study of healthy young male volunteers.

REFERENCES

1 J.M. Adam, D.J. Bennett, A. Bom, J.K. Clark, *et al., Cyclodextrin-derived host molecules as reversal agents for the neuromuscular blocker rocuronium bromide: synthesis and structure-activity relationships.* J Med Chem, 2002. 45(9): p. 1806-1816.

2 A. Bom, M. Bradley, K. Cameron, J.K. Clark, *et al., A novel concept of reversing neuromuscular block: chemical encapsulation of rocuronium bromide by a cyclodextrin-based synthetic host.* Angew Chem Int Ed Engl, 2002. 41(2): p. 266-270.

3 M. Blobner, L.I. Eriksson, J. Scholz, J. Motsch, *et al., Reversal of rocuronium-induced neuromuscular blockade with sugammadex compared with neostigmine during sevoflurane anaesthesia: results of a randomised, controlled trial.* Eur J Anaesthesiol, 2010. 27(10): p. 874-881.

4 R.K. Jones, J.E. Caldwell, S.J. Brull, and R.G. Soto, *Reversal of profound rocuronium-induced blockade with sugammadex: a randomized comparison with neostigmine.* Anesthesiology, 2008. 109(5): p. 816-824.

5 K.S. Khuenl-Brady, M. Wattwil, B.F. Vanacker, J.I. Lora-Tamayo, *et al., Sugammadex provides faster reversal of vecuronium-induced neuromuscular blockade compared with neostigmine: a multicenter, randomized, controlled trial.* Anesth Analg, 2010. 110(1): p. 64-73.

6 H.J. Lemmens, M.I. El-Orbany, J. Berry, J.B. Morte, Jr., *et al., Reversal of profound vecuronium-induced neuromuscular block under sevoflurane anesthesia: sugammadex versus neostigmine.* BMC Anesthesiol, 2010. 10(15): p. 1-10.

7 N. Rahe-Meyer, S. Schulman, W. Klimscha, M. Przemeck, *et al., Sugammadex reversal of rocuronium- or vecuronium-induced neuromuscular blockade is not associated with an increased risk of either bleeding or blood loss compared with usual care.* Abstract submitted to: The International Anesthesia Research Society (IARS) Annual Meeting 2013, San Diego, California.

8 A. Soni, *Aspirin use among the adult U.S. noninstitutionalized population, with and without indicators of heart disease, 2005.* Statistical brief #179. Agency for Healthcare Research and Quality, Rockville, MD. Available from: http://meps.ahrq.gov/mepsweb/data_files/publications/st179/stat179.pdf.

9 U.A. Ajani, E.S. Ford, K.J. Greenland, W.H. Giles, *et al., Aspirin use among U.S. adults: Behavioral Risk Factor Surveillance System.* Am J Prev Med, 2006. 30(1): p. 74-77.

10 M. Moerland, M.J. Kemme, M. van der Linden, and J. Burggraaf, *Measurement of collagen- and serotonin-induced platelet aggregation in whole blood.* Expert Rev Clin Pharmacol, 2010. 3(2): p. 177-182.

11 C. Patrono, L.A. Garcia Rodriguez, R. Landolfi, and C. Baigent, *Low-dose aspirin for the prevention of atherothrombosis.* N Engl J Med, 2005. 353(22): p. 2373-2383.

12 H. Caplain, *Enhanced antiplatelet effects of clopidogrel plus acetylsalicylic acid compared with acetylsalicylic acid alone or combined with extended-release dipyridamole in healthy volunteers.* Cerebrovasc Dis, 2005. 19(4): p. 214-219.

13 T. Yokoyama, F. Yamasaki, K. Yamashita, M. Manabe, *et al., Bleeding time prolonged by daily low-dose aspirin is shortened by one medium dose aspirin.* Acta Anaesthesiol Scand, 2008. 52(9): p. 1226-1230.

14 T. Koster, K.M. Caekebeke-Peerlinck, and E. Briet, *A randomized and blinded comparison of the sensitivity and the reproducibility of the Ivy and Simplate II bleeding time techniques.* Am J Clin Pathol, 1989. 92(3): p. 315-320.

15 M.A. de Zwart, J. ten Bruggencate-Broeders, H.J. van Hal, R.H. Megens, *et al., Determination of sugammadex in human plasma, urine, and dialysate using a high-performance liquid chromatography/tandem mass spectrometry assay.* J Chromatogr B Analyt Technol Biomed Life Sci, 2011. 879(19): p. 1573-1586.

16 U. Klinkhardt, C.M. Kirchmaier, D. Westrup, J. Graff, *et al., Ex vivo - in vitro interaction between aspirin, clopidogrel, and the glycoprotein IIb/IIIa inhibitors abciximab and SR121566A.* Clin Pharmacol Ther, 2000. 67(3): p. 305-313.

17 S. Saha, M. Berglund, C. Sylvén, A. Gordon, *et al., Clopidogrel inhibits platelet aggregation in patients on aspirin with stable chronic angina pectoris.* Int J Cardiol, 2008. 123(2): p. 195-196.

18 M.A. Mascelli, S. Worley, N.J. Veriabo, E.T. Lance, *et al., Rapid assessment of platelet function with a modified whole-blood aggregometer in percutaneous transluminal coronary angioplasty patients receiving anti-GP IIb/IIIa therapy.* Circulation, 1997. 96(11): p. 3860-3866.

19 P.J. de Kam, R. El Galta, M.J. van Lierop, J. Dennie, *et al., Sugammadex (4 mg/kg or 16 mg/kg) has no clinically relevant interaction effects on anticoagulant activity of enoxaparin or unfractionated heparin.* Abstract submitted to: The International Anesthesia Research Society (IARS) Annual Meeting 2013, San Diego, California.

20 D.I. Jalal, M. Chonchol, and G. Targher, *Disorders of hemostasis associated with chronic kidney disease.* Semin Thromb Hemost, 2010. 36(1): p. 34-40.

21 P.R. Lichtenthal, E.C. Rossi, G. Louis, K.A. Rehnberg, *et al., Dose-related prolongation of the bleeding time by intravenous nitroglycerin.* Anesth Analg, 1985. 64(1): p. 30-33.

22 I.P. Zupan, M. Šabovič, B. Salobir, J.B. Ponikvar, *et al., Utility of in vitro closure time test for evaluating platelet-related primary hemostasis in dialysis patients.* Am J Kidney Dis, 2003. 42(4): p. 746-751.

Figure 1 Study design.

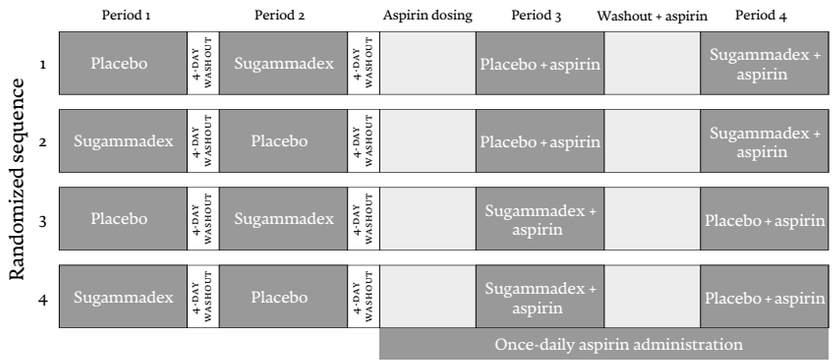
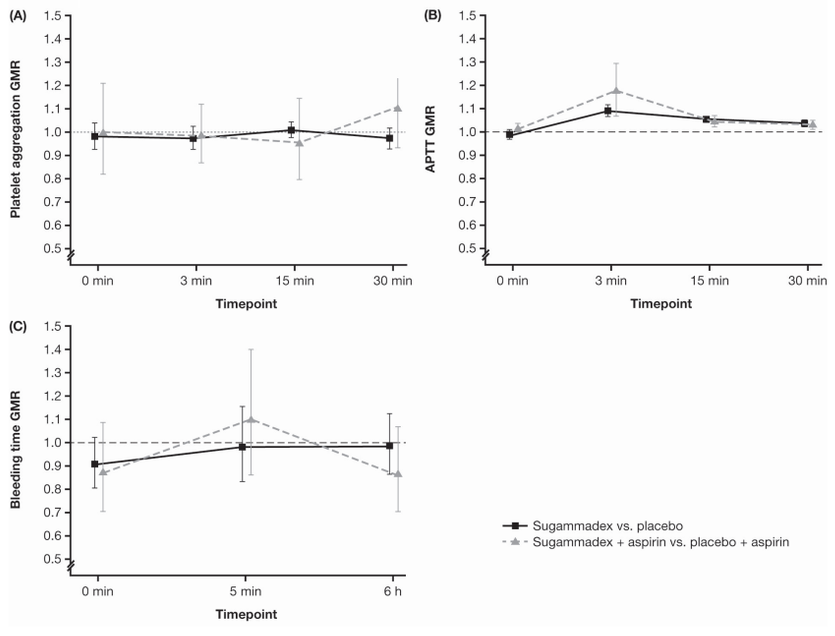
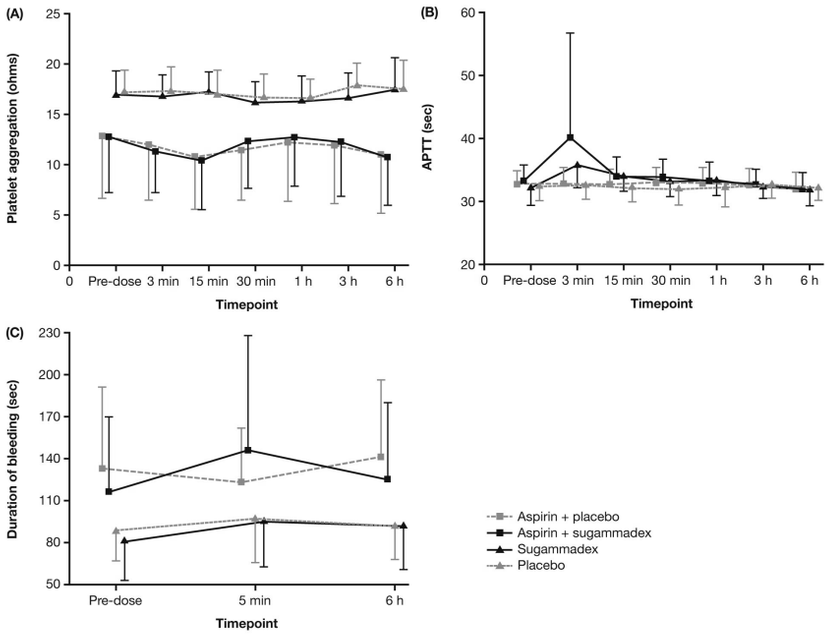


Figure 2 Geometric mean ratio and corresponding limits of the two-sided 90% CI by time point for (A) platelet aggregation, (B) activated partial thromboplastin time (APTT), and (C) bleeding time.



APTT = activated partial thromboplastin time; CI = confidence interval; and GMR = geometric mean ratio.

Figure 3 Mean (SD) values by time point for (A) platelet aggregation, (B) activated partial thromboplastin time (APTT), and (C) bleeding time.



APTT = activated partial thromboplastin time; and SD = standard deviation.

Table 1 Geometric mean ratios, with relevant limits of the two-sided 90% confidence interval (CI), and non-inferiority margin for platelet aggregation, activated partial thromboplastin time (APTT), and bleeding time (AST group, n = 26).

Parameter	Treatment comparison	Geometric mean ratio	Relevant limit of the two-sided 90% CI[a]	Non-inferiority margin
Whole blood platelet aggregation[b]	Sugammadex + aspirin *versus* aspirin alone	1.01	0.91	> 0.75
	Sugammadex alone *versus* placebo alone	0.99	0.96	
	Statistical interaction between sugammadex and aspirin	1.02	0.91	
APTT[b]	Statistical interaction between sugammadex and aspirin	1.01	1.04	< 1.5
	Sugammadex alone *versus* placebo alone	1.06	1.07	< 1.5
	Sugammadex + aspirin *versus* aspirin alone	1.07	1.10	
Bleeding time (at 5 minutes after study drug)	Sugammadex + aspirin *versus* aspirin alone	1.20	1.45	< 1.5
	Sugammadex alone *versus* placebo alone	1.08	1.28	
	Statistical interaction between sugammadex and aspirin	1.12	1.44	

(a) Lower limit of the two-sided 90% CI for platelet aggregation and upper limit for APTT and bleeding time; and (b) determined as the area under the exposure curve between 3 and 30 minutes divided by the time span. APTT = activated partial thromboplastin time; AST = all-subjects-treated; and CI = confidence interval.

Table 2 Geometric mean values (and CV%) of pharmacokinetic parameters for sugammadex when given alone and in combination with aspirin (all-subjects-pharmacokinetically-evaluable group).

	Sugammadex (n = 23)	Sugammadex + aspirin (n = 23)
C_{max}, µg/mL	45.0 (26.6)	44.9 (19.2)
AUC_{0-6h}, µg*h/mL	34.8 (15.9)	35.9 (14.9)

AUC_{0-6h} = area under the concentration-time curve from 0 to 6 hours; C_{max} = maximum plasma concentration; and CV = coefficient of variation assuming log-normality.

CHAPTER 6

Lack of a clinically relevant effect of sugammadex on anti-Xa activity or activated partial thromboplastin time following pretreatment with either unfractionated or low-molecular-weight heparin in healthy subjects

International Journal of Clinical Pharmacology and Therapeutics, 2014. 52(8): p. 631-641, doi: 10.5414/CP202091

Pieter-Jan de Kam, Annelieke C. Kruithof, Marie-José van Lierop, Matthijs Moerland, Justin Dennie, Matthew D. Troyer, Ronald B. Langdon, David E. Gutstein, Jacobus Burggraaf, and Rachid El Galta

Abstract

OBJECTIVE To investigate the potential effect of sugammadex on anti-Xa anticoagulant activity of enoxaparin and the activated partial thromboplastin time (APTT) of unfractionated heparin (UFH).

METHODS This two-part, randomized, double-blind, placebo-controlled, four-period cross-over study was performed in healthy males (18–45 years). In each period, subjects received 40 mg enoxaparin (in Part 1), 5,000 units UFH (in Part 2), or placebo followed by 4 or 16 mg/kg sugammadex, or placebo. Treatments were separated by ≥ 4 days. Primary endpoints were anti-Xa activity and APTT both time-averaged from 3 to 30 minutes post-dose. Geometric mean ratios (GMRs) and their two-sided 90% confidence limits were calculated for anticoagulant plus sugammadex (4 or 16 mg/kg) *versus* anticoagulant plus placebo. The pre-specified threshold for a potential effect of clinical relevance was a 90% upper confidence limit (UCL) > 1.50.

RESULTS In Part 1 (n = 13), the 90% UCLs were 1.07 and 1.08 for GMRs of anti-Xa activity after dosing with 4 and 16 mg/kg sugammadex, respectively. In Part 2 (n = 43), the 90% UCLs for GMRs of APTT were 1.06 and 1.15. Neither sugammadex dose produced a treatment effect that met the pre-specified criterion for potential clinical relevance. Treatments were generally well-tolerated.

CONCLUSIONS In healthy subjects, treatment with 4 mg/kg and 16 mg/kg sugammadex did not change either anti-Xa activity or APTT to a clinically meaningful extent following pretreatments with enoxaparin or UFH.

Introduction

Sugammadex is a modified γ-cyclodextrin that selectively encapsulates the steroidal neuromuscular blocking agents rocuronium and vecuronium and thereby reverses neuromuscular blockade (NMB) induced by these agents.[1,2] Sugammadex has been widely approved for post-surgical use since 2008. For routine reversal of NMB, it is administered in doses of 2 or 4 mg/kg and this generally results in recovery within 2–3 minutes.[3] Sugammadex may also be administered at a higher dose (16 mg/kg) when there is a need for immediate reversal of NMB in emergency situations.

Prior *in vitro* investigation has shown that sugammadex increases activated partial thromboplastin time (APTT) and prothrombin time (PT) in a concentration-dependent manner. When sugammadex is added to normal human plasma at a concentration of 200 μg/mL (approximately the mean maximum concentration that is reached *in vivo* after a dose of 16 mg/kg sugammadex), APTT and PT are increased by 20–30%.[4] A clinical study in healthy subjects showed that administration of 16 mg/kg sugammadex results in transient increases of a similar magnitude in APTT and international normalized ratio for PT (PT(INR)).[4] In addition, transient increases of 5–10% have been observed in healthy subjects and surgical patients after administration of 4 mg/kg sugammadex.[4–6] These increases in APTT and PT(INR) alone are unlikely to be clinically relevant because they are brief in duration and relatively small. There is a need, nonetheless, to assess whether sugammadex may possibly interact with anticoagulants that are administered clinically and thereby have larger effects that could be clinically relevant.

The goal of the present study (performed in two parts) was to investigate the potential effect of sugammadex on either the anti-Xa anticoagulant activity of enoxaparin (a low-molecular-weight heparin) or on the APTT of unfractionated heparin (UFH), both of which are currently used perioperatively for prophylaxis of thrombosis. In Part 1 of this study, subjects were pretreated with enoxaparin or placebo and subsequently treated with sugammadex or placebo. Plasma anti-Xa activity is generally recognized as a sensitive parameter to measure the anticoagulant activity of enoxaparin and was considered the endpoint of principal interest.[7–10] In Part 2, subjects were pretreated with UFH or placebo and APTT was studied as the endpoint of principal interest because anticoagulation status in patients treated with UFH is commonly monitored by measuring APTT.[11,12] APTT and anti-Xa activity were

secondary endpoints in Parts 1 and 2, respectively, and PT(INR) was an exploratory endpoint in both parts of this study. Exploratory analysis of pharmacokinetic/pharmacodynamic (PK/PD) relationships was included to support further understanding of potential effects of sugammadex on anti-Xa activity, APTT, and PT(INR).

Methods

Parts 1 and 2 of this study (Protocol P07044) were separate, randomized, double-blind, placebo-controlled, cross-over trials that included four periods. The trials were performed between October 5, 2011 and 20 December, 2011 at the Centre for Human Drug Research (CHDR) in Leiden, The Netherlands. The protocol was reviewed and approved by an independent ethics committee at the Leiden University Medical Center (Leiden, The Netherlands) prior to conducting the study. All subjects provided written informed consent prior to their participation and the study was performed in accordance with the principles of Good Clinical Practice.

INCLUSION/EXCLUSION CRITERIA

Eligible subjects were male, 18–45 years of age, and had a body mass index between 18 and 32 kg/m^2, inclusively. They were in good health as confirmed within 3 weeks prior to randomization by medical history, physical examination, electrocardiography, laboratory analysis of blood, and urinalysis. At screening, subjects were required to have plasma anti-Xa activity below 0.03 IU/mL, the limit of quantitation (LOQ), and APTT and PT(INR) values within normal limits. Co-medication was prohibited from 21 days prior to the first dose of study drug through the end of the study.

SPECIFIC PROCEDURES

In each study period, subjects were pretreated with a single subcutaneous abdominal injection of anticoagulant (40 mg enoxaparin in Part 1, 5,000 units of UFH in Part 2) or placebo (isotonic saline) followed 3 hours later by a single intravenous dose of 4 mg/kg or 16 mg/kg sugammadex, or placebo (saline) delivered as a 10 seconds bolus. The doses of enoxaparin and UFH were consistent with standard guidelines for thromboprophylactic care in surgical patients.[13,14] Sugammadex or placebo was administered 3 hours after pretreatments to allow time for the anticoagulants to reach near-maximal activity.[8–10,12] Enoxaparin (Clexane®) was obtained from Sanofi Aventis, Gouda, The Netherlands, and UFH (Heparin LEO®) from Leo Pharma BV. Sugammadex (Bridion®), Schering-Plough, Oss, The Netherlands, was provided for this study by the sponsor.

In Part 1, each subject was randomly assigned to receive one of the following treatment combinations (Figure 1A): enoxaparin placebo plus sugammadex 4 mg/kg, enoxaparin plus sugammadex placebo, enoxaparin plus sugammadex 4 mg/kg, and enoxaparin plus sugammadex 16 mg/kg. Part 2 was similar, except that UFH 5,000 units replaced enoxaparin and pretreatment with the UFH placebo was followed treatment with sugammadex 16 mg/kg (Figure 1B). Treatments were separated by a minimum of 4 days.

Blood samples for PD and PK analysis were obtained via an indwelling catheter placed in a forearm vein. For analysis of anti-Xa activity and APTT, blood was collected prior to dosing with anticoagulant or placebo (t = –180 minutes), at 6 subsequent timepoints prior to dosing with sugammadex or its placebo (t = –150 to –5 minutes), and at 7 timepoints following such dosing (t = 3 to 360 minutes). Blood was obtained for PK analysis and analysis of PT(INR) 5 minutes prior to dosing with sugammadex/placebo and at 7 timepoints thereafter until 360 minutes post-dose. The samples for PD analysis were collected into vacuum tubes containing citrate, theophylline, adenosine, and dipyridamole. Plasma was then obtained by centrifugation and stored frozen until analyzed.

BIOANALYTICAL PROCEDURES

All PD analyses were performed at Good Biomarker Sciences (Leiden, The Netherlands). Anti-Xa activity was assessed using a Coamatic® heparin kit (Chromogenix, Milan, Italy) and STA® analyzer (PerkinElmer, Waltham, Massachusetts, United States of America (USA)). Assays of APTT and PT(INR) were performed using an AMAX Destiny Plus automated clotting analyzer (Fisher Scientific, Pittsburgh, Pennsylvania, USA) and, respectively, Triniclot Automated APTT and Triniclot PT HTF as reagent kits (Trinity Biotech, Kordia Lifesciences, Leiden, The Netherlands). PK analysis of plasma sugammadex was performed at the Department of Bioanalytics-Oss, MSD (Oss, The Netherlands) using a validated liquid chromatographic assay with mass spectrometric detection as described previously,[15] with the following modifications. The sample preparation method was changed from solid phase extraction to protein precipitation, in which the sample volume was reduced from

250 µL to 100 µL; the mass spectrometer was changed from AB SCIEX API3000 to API 5000; the analytical column was upgraded from a Varian Polaris 5 C18 A column (4.6 × 50 mm, 5 µm) to Waters BEH Shield RP18 column (2.1 × 50 mm, 1.7 µm) and to minimize carryover, the mobile phase was changed from a gradient of 0.1% formic acid aqueous solution (A) and methanol (B) to a gradient of 50% (v/v) methanol/water, 10 mM NH4FA (pH = 3.0) (A) and 85% (v/v) ACN/water, 10 mM NH4FA (pH = 3.0) (B).

STATISTICAL ANALYSES

The primary PD analyses included all randomized subjects for whom there was at least one post-dose measurement of the endpoint being evaluated. The primary time interval of interest for assessment of treatment effects was pre-specified as 3–30 minutes post-dose following administration of sugammadex or its placebo. The primary endpoint in Part 1 was anti-Xa activity time-averaged within this interval (anti-Xa activity$_{3–30\,min}$); in Part 2, it was APTT time-averaged within this interval (APTT$_{3–30\,min}$). These endpoints were evaluated using a mixed model for repeated measures in which the log of the endpoint was the dependent variable and treatment and period were fixed factors. In primary comparisons, this model included log baseline as a covariate and a treatment factor with three categories (treatment with anticoagulant followed by 4 mg/kg sugammadex, 16 mg/kg sugammadex, or sugammadex placebo). Baseline anti-Xa activity and baseline APTT were determined by averaging measurements made over the interval from 5 to 30 minutes pre-dose to sugammadex or its placebo. Geometric mean ratios (GMRs) and two-sided 90% confidence intervals (CIs) were calculated to evaluate differences in response to treatment. It was pre-specified that a treatment effect would not be regarded as having potential clinical relevance unless comparison between active treatment and placebo resulted in a GMR with a 90% upper confidence limit (UCL) > 1.50. This threshold was derived by consideration of anticoagulation levels that are targeted clinically to provide thromboprophylaxis with little or no increased bleeding risk.[16–19] In Part 1, the null hypothesis (a joint hypothesis) was that the GMR does not have a 90% UCL > 1.50 in either of two comparisons: (1) enoxaparin plus 4 mg/kg sugammadex *versus* enoxaparin plus sugammadex placebo and (2) enoxaparin plus 16 mg/kg sugammadex *versus* enoxaparin plus sugammadex placebo. In Part 2, the joint null hypothesis was the same except that UFH substituted for enoxaparin. Supportive analyses were performed in which treatment effects were evaluated from 3 to 360 minutes post-dose to sugammadex or its placebo. These included evaluations of both mean and maximum values for anti-Xa activity and APTT in this interval (mean and maximum anti-Xa activity$_{3–360\,min}$ and APTT$_{3–360\,min}$, respectively).

PK data were analyzed non-compartmentally using SAS version 9.1.3. Exploratory PK/PD analyses included data collected between 5 minutes pre-dose to sugammadex or its placebo until 30 minutes post-dose, an interval during which plasma concentrations of enoxaparin and UFH were expected to be relatively constant.

DETERMINATION OF SAMPLE SIZE AND PLANNED STATISTICAL POWER

In Part 1, initial enrollment of 12 subjects was planned with the expectation that this would provide ≥ 83% power to reject the joint null hypothesis at a one-sided significance level of 5%, assuming that the true GMRs for mean anti-Xa activity$_{3–30\,min}$ in primary comparisons were ≤ 1.2 and that within-subject coefficients of variation (CVs) for mean anti-Xa activity$_{3–30\,min}$ were ≤ 17%. In Part 2, initial enrollment of 40 subjects was planned, expecting that this would provide ≥ 79% power to reject the joint null hypothesis assuming that the true GMRs for mean APTT$_{3–30\,min}$ in the primary comparisons were ≤ 1.2 (for UFH plus 4 mg/kg sugammadex *versus* UFH plus sugammadex placebo) and ≤ 1.3 (for UFH plus 16 mg/kg sugammadex *versus* UFH plus sugammadex placebo), and that within-subject CV for mean APTT$_{3–30\,min}$ was ≤ 25%.

The protocol allowed replacement of discontinued subjects and specified that additional subjects could be enrolled depending on the outcome of blinded interim analyses of within-subject variability. These interim analyses found that the within-subject CV was < 13% in Part 1 and < 9% in Part 2. Thus, the initial assumptions regarding within-subject variability were validated and no additional subjects were enrolled.

Results

Thirteen subjects were randomized in Part 1 and 43 in Part 2. Baseline characteristics of the randomized populations are summarized in Table 1. A total of 12 of the 13 and 39 of the 43 subjects completed all 4 study periods in Parts 1 and 2, respectively.

ANTI-XA ACTIVITY IN PART 1

Anti-Xa activity was generally undetectable (below the LOQ of 0.03 IU/mL) before dosing with enoxaparin and following pretreatment with the enoxaparin placebo. After treatment with enoxaparin, anti-Xa activity increased for the first 2–3 hours and declined thereafter (Figure 2A). Similar levels of anti-Xa activity were observed after administration of 4 mg/kg sugammadex, 16 mg/kg sugammadex, and sugammadex placebo (mean anti-Xa activity$_{3-30\ min}$ = 0.31–0.32 IU/mL; Table 2). The 90% UCL of the GMR for mean anti-Xa activity$_{3-30\ min}$ of enoxaparin plus 4 mg/kg sugammadex *versus* enoxaparin plus sugammadex placebo was 1.07 (Table 3). Comparing enoxaparin plus 16 mg/kg sugammadex with enoxaparin plus sugammadex placebo, the corresponding 90% UCL was 1.08. Both of these values were far below the pre-specified threshold for clinical relevance (90% UCL > 1.50).

Findings from the supportive analyses of maximum and mean anti-Xa activity$_{3-360\ min}$ were consistent with the results of the primary analysis. Mean anti-Xa activity$_{3-360\ min}$ after dosing with enoxaparin was 0.20–0.21 IU/mL, regardless of whether subjects were then treated with 4 mg/kg sugammadex, 16 mg/kg sugammadex, or sugammadex placebo. Geometric means (95% CIs) for maximum anti-Xa activity$_{3-360\ min}$ were 0.31 (0.25–0.39), 0.36 (0.29–0.45), and 0.36 (0.29–0.44) IU/mL following treatments with enoxaparin plus sugammadex placebo, enoxaparin plus 4 mg/kg sugammadex, and enoxaparin plus 16 mg/kg sugammadex, respectively.

OTHER PD OBSERVATIONS IN PART 1

Treatment with sugammadex elicited small, transient, dose-dependent increases in APTT and PT(INR) that were maximal at 3 minutes post-dose and declined rapidly thereafter (Figure 2B and C). Based on the GMRs derived from comparisons between enoxaparin plus sugammadex treatment and enoxaparin plus sugammadex placebo, treatment with sugammadex increased mean APTT$_{3-30\ min}$ by 2–9% in subjects pretreated with enoxaparin (Table 3).

The 90% UCL of the GMR for mean APTT$_{3-30\ min}$ of enoxaparin plus 4 mg/kg sugammadex *versus* enoxaparin plus sugammadex placebo was 1.05 (Table 3). The 90% UCL of the GMR for mean APTT$_{3-30\ min}$ of enoxaparin plus 16 mg/kg sugammadex *versus* enoxaparin plus sugammadex placebo was 1.13. The GMR for mean APTT$_{3-30\ min}$ of enoxaparin plus 4 mg/kg sugammadex *versus* enoxaparin placebo plus 4 mg/kg sugammadex was 1.19 (90% CI, 1.14–1.24). The effect of sugammadex on PT(INR) was not affected by pretreatment with enoxaparin (Figure 2C).

APTT IN PART 2

Before dosing with UFH in Part 2, values for APTT were generally between 30 and 35 seconds. Following administration of UFH, mean APTT generally increased by 2–3 seconds (Figure 2E). Following pretreatments with either UFH or the UFH placebo, sugammadex elicited small, transient, dose-dependent increases in APTT in Part 2 similar to those observed in Part 1 (compare Figure 2E with 2B). Following treatment with UFH plus the sugammadex placebo, the mean APTT$_{3-30\ min}$ was 33.3 seconds (Table 2). Following treatments with UFH plus 4 mg/kg sugammadex and UFH plus 16 mg/kg sugammadex, mean APTT$_{3-30\ min}$ increased to 34.8 and 37.8 seconds, respectively. The 90% UCL of the GMR for mean APTT$_{3-30\ min}$ of UFH plus 4 mg/kg sugammadex *versus* UFH plus sugammadex placebo was 1.06. The 90% UCL of the GMR for mean APTT$_{3-30\ min}$ of UFH plus 16 mg/kg sugammadex *versus* UFH plus sugammadex placebo was 1.15. As in Part 1, these values were far below the pre-specified threshold for clinical relevance (90% UCL > 1.50).

The GMR for mean APTT$_{3-30\ min}$ from comparison of UHF plus 16 mg/kg sugammadex treatment with UFH placebo plus 16 mg/kg sugammadex treatment was 1.07 (90% CI, 1.05–1.09).

The supportive analyses of maximum and mean APTT$_{3-360\ min}$ showed results consistent with the primary analysis. Geometric mean APTT$_{3-360\ min}$ was 32.5 seconds following treatment with UFH placebo plus 16 mg/kg sugammadex, and also 32.5 seconds following treatment with UFH plus sugammadex placebo. It was 33.1 seconds following treatment with UFH plus 4 mg/kg sugammadex and 33.6 seconds following treatment with UFH plus 16 mg/kg sugammadex.

OTHER PD OBSERVATIONS IN PART 2

Pretreatment with UFH appeared to increase anti-Xa activity to a small extent (Table 2 and Figure 2D). As in Part 1, sugammadex had no apparent effect on anti-Xa activity.

Sugammadex treatment elicited a small, transient increase in PT(INR) in Part 2 similar to that observed Part 1 (compare Figures 2C and 2F). Treatment

with UFH appeared to have no relevant effect on PT(INR), nor did it modify the limited effect of sugammadex on PT(INR).

PK RESULTS AND PK/PD ANALYSIS

Pretreatment with enoxaparin or UFH appeared to have no effect on the PK profile of sugammadex and values for sugammadex maximum plasma concentration (C_{max}) and area under the concentration-time curve from 0 to 6 hours ($AUC_{0-6\ h}$) were similar, regardless of pretreatment condition (Figure 3). Exploratory PK/PD analysis of the data from 3–30 minutes post-dose to sugammadex supported the conclusion that sugammadex had no effect on anti-Xa activity, regardless of pretreatment. Following pretreatment with enoxaparin, individual values for anti-Xa activity were generally in the range 0.2–0.6 IU/mL (Figure 4). Following pretreatment with UFH, they were generally < 0.1 IU/mL, and following pretreatment with UFH placebo, they were generally below the LOQ.

Analyses of APTT and PT(INR) *versus* plasma sugammadex concentration suggested a positive concentration-effect relationship. Based on linear regression analysis, APTT tended to increase by 0.023–0.032 second and PT(INR) by 0.0011–0.0013 unit per 1 µg/mL increase in plasma sugammadex. Based on y-intercept differences, pretreatment with enoxaparin increased APTT by 17% and pretreatment with UFH increased APTT by 6%, relative to placebo. Neither of these pretreatments appeared to modify PT(INR).

SAFETY AND TOLERABILITY

Treatment-emergent adverse events (TEAEs) were reported in all 13 subjects in Part 1 and in 41 (95%) of the 43 subjects in Part 2. None of these was serious and all were mild in intensity. Combining the data from Parts 1 and 2 (n = 56), the most frequently reported TEAEs were dysgeusia, administration site reaction, nausea, headache, nasopharyngitis, and dizziness. These occurred in 21 (38%), 16 (29%), 13 (23%), 12 (21%), 11 (20%), and 9 (16%) subjects, respectively. TEAEs of dysgeusia and nausea were generally regarded by the investigator as sugammadex-related. TEAEs of administration site reaction were generally regarded by the investigator as possibly or probably related to treatments with anticoagulant.

One subject was discontinued in Part 1 because of TEAEs of discolored feces and abnormal urine color (both regarded by the investigator as possibly related to sugammadex treatment). Three subjects were discontinued in Part 2 because of administration site reactions that followed anticoagulant injections. One subject was discontinued in Part 2 because of a TEAE of hemorrhoids, first noted one day after dosing with UFH and 4 mg/kg sugammadex.

Discussion

Previous studies showed that treatment with sugammadex resulted in small, transient increases in APTT and PT(INR).[4,5] Additional *in vitro* experiments suggested that these effects may be secondary to direct, concentration-dependent inhibition of factor Xa generation and activity. The primary purpose of the present study was to evaluate potential effects of 4 mg/kg and 16 mg/kg sugammadex on anti-Xa activity following pretreatment with enoxaparin and APTT activity following pretreatment with UFH. Enoxaparin and UFH are both anticoagulants commonly administered to surgical patients for thromboprophylaxis. The potential interaction between sugammadex and these anticoagulants was tested ≈3 hours after the anticoagulants were administered. It was expected that enoxaparin and UFH would reach near-maximal PD effects at this time and this expectation was confirmed by the data.

The present findings indicate that treatment with sugammadex, following pretreatment with enoxaparin or UFH, does not increase anti-Xa activity or APTT to a clinically meaningful extent. Sugammadex did elicit small, transient, dose-dependent effects on APTT. The 90% UCLs for the GMRs for mean $APTT_{3-30\ min}$ increase relative to sugammadex placebo were 5–6% and 13–15% after 4 mg/kg and 16 mg/kg sugammadex, respectively. These increases were similar to those observed in previous studies.[4,5] The maximum APTT and PT(INR) increases measured immediately after dosing with 16 mg/kg sugammadex were ≈25%, returning rapidly to near-pretreatment levels within 30–60 minutes. The time course of these increases was consistent with the known PK behavior of sugammadex; it is rapidly distributed and cleared from the blood following intravenous dosing (Figure 3; see also references 20 and 21[20,21]).

In evaluating the potential clinical relevance of such increases, consideration must be given to both their magnitude and duration. In clinical practice, thromboprophylactic guidelines call for treatments that provide sustained 1.5- to 3.0-fold increases in APTT[16] and 2.0- to 3.0-fold or 3.5-fold increases in PT(INR).[17–19] In the present study, the duration of effect was factored into the consideration of potential clinical relevance by evaluating

anti-Xa activity and APTT integrated over the interval of 3–30 minutes post-dose. Times later than 30 minutes may reasonably be regarded as irrelevant because little sugammadex remains in blood after 30 minutes. In this study, all comparisons between active treatment and placebo resulted in GMRs for anti-Xa activity$_{3–30\ min}$ and APTT$_{3–30\ min}$ with 90% UCLs far below any level that could be regarded as potentially clinically relevant.

As a further consideration, it may be noted that the transient increases in APTT and PT(INR) observed in this study and in previous studies were primarily the result of treatment with 16 mg/kg sugammadex. This is a dose that is only recommended for use in situations in which there is an urgent need for immediate reversal of NMB. Under such circumstances, surgery will generally be postponed and the potential relevance of brief increases in APTT and PT(INR) will thus be further diminished.

One limitation of the present study was that coagulation status was assessed using coagulation laboratory parameters and not directly through measurement of bleeding events. However, a recent large clinical study showed that sugammadex treatment does not increase the incidence of bleeding or blood loss in surgical patients who were treated with anticoagulants for thromboprophylaxis.[6]

Exposure to sugammadex was generally well tolerated in this study. Dysgeusia was the most frequently observed adverse event after 16 mg/kg sugammadex and was previously reported at higher sugammadex doses in conscious healthy subjects.[22]

Conclusion

In this study, treatment of healthy subjects with 4 mg/kg or 16 mg/kg sugammadex did not change either anti-Xa activity or APTT to a clinically meaningful extent following pretreatment with enoxaparin or UFH.

REFERENCES

1 J.M. Adam, D.J. Bennett, A. Bom, J.K. Clark, *et al., Cyclodextrin-derived host molecules as reversal agents for the neuromuscular blocker rocuronium bromide: synthesis and structure-activity relationships.* J Med Chem, 2002. 45(9): p. 1806-1816.

2 L.P. Yang and S.J. Keam, *Sugammadex: a review of its use in anaesthetic practice.* Drugs, 2009. 69(7): p. 919-942.

3 European Medicines Agency. *Bridion Annex I: Summary of Product Characteristics*. Available from: http://www.ema.europa.eu/docs/en_GB/document_library/EPAR_-_Product_Information/human/000885/WC500052310.pdf.

4 P.J. de Kam, P. Grobara, M. Prohn, F. Höppener, *et al., Effects of sugammadex on activated partial thromboplastin time and prothrombin time in healthy subjects.* Int J Clin Pharmacol Ther, 2014. 52(3): p. 227-236.

5 P.J. de Kam, R. El Galta, A.C. Kruithof, H. Fennema, *et al., No clinically relevant interaction between sugammadex and aspirin on platelet aggregation and coagulation parameters.* Int J Clin Pharmacol Ther, 2013. 51(12): p. 976-985.

6 N. Rahe-Meyer, H. Fennema, S. Schulman, W. Klimscha, *et al., Effect of reversal of neuromuscular blockade with sugammadex versus usual care on bleeding risk.* Anesthesiology. Submitted.

7 C.A. Carter, V.A. Skoutakis, T.E. Spiro, M.E. West, *et al., Enoxaparin: the low-molecular-weight heparin for prevention of postoperative thromboembolic complications.* Ann Pharmacother, 1993. 27(10): p. 1223-1230.

8 J. Fareed, D. Hoppensteadt, J. Walenga, O. Iqbal, *et al., Pharmacodynamic and pharmacokinetic properties of enoxaparin: implications for clinical practice.* Clin Pharmacokinet, 2003. 42(12): p. 1043-1057.

9 A.M. Frydman, L. Bara, Y. Le Roux, M. Woler, *et al., The antithrombotic activity and pharmacokinetics of enoxaparine, a low molecular weight heparin, in humans given single subcutaneous doses of 20 to 80 mg.* J Clin Pharmacol, 1988. 28(7): p. 609-618.

10 K. Kuczka, S. Harder, B. Picard-Willems, A. Warnke, *et al., Biomarkers and coagulation tests for assessing the biosimilarity of a generic low-molecular-weight heparin: results of a study in healthy subjects with enoxaparin.* J Clin Pharmacol, 2008. 48(10): p. 1189-1196.

11 C. Kearon, L. Harrison, M. Crowther, and J.S. Ginsberg, *Optimal dosing of subcutaneous unfractionated heparin for the treatment of deep vein thrombosis.* Thromb Res, 2000. 97(6): p. 395-403.

12 C. Kroon, W.R. ten Hove, A. de Boer, J.M. Kroon, *et al., Highly variable anticoagulant response after subcutaneous administration of high-dose (12,500 IU) heparin in patients with myocardial infarction and healthy volunteers.* Circulation, 1992. 86(5): p. 1370-1375.

13 Sanofi-Aventis U.S. LLC. *Lovenox [Prescribing Information]*. 2013; Available from: http://products.sanofi.us/lovenox/lovenox.html.

14 Society of American Gastrointestinal and Endoscopic Surgeons. *Guidelines for deep venous thrombosis prophylaxis during laparoscopic surgery*. Available from: Society of American Gastrointestinal and Endoscopic Surgeons (SAGES) Website. Guidelines for deep venous thrombosis prophylaxis during laparoscopic surgery. http://www.sages.org/publications/guidelines/guidelines-for-deepvenous-thrombosis-prophylaxis-during-laparoscopic-surgery.

15 M.A. de Zwart, J. ten Bruggencate-Broeders, H.J. van Hal, R.H. Megens, *et al., Determination of sugammadex in human plasma, urine, and dialysate using a high-performance liquid chromatography/tandem mass spectrometry assay.* J Chromatogr B Analyt Technol Biomed Life Sci, 2011. 879(19): p. 1573-1586.

16 A.D. Blann and C.W. Khoo, *The prevention and treatment of venous thromboembolism with LMWHs and new anticoagulants.* Vasc Health Risk Manag, 2009. 5: p. 693-704.

17 R.O. Bonow, B.A. Carabello, K. Chatterjee, A.C. de Leon Jr., *et al., 2008 Focused update incorporated into the ACC/AHA 2006 guidelines for the management of patients with valvular heart disease: a report of the American College of Cardiology/American Heart Association Task Force on Practice Guidelines (Writing Committee to revise the 1998 guidelines for the management of patients with valvular heart disease): endorsed by the Society of Cardiovascular Anesthesiologists, Society for Cardiovascular Angiography and Interventions, and Society of Thoracic Surgeons.* Circulation, 2008. 118(15): p. e523-661.

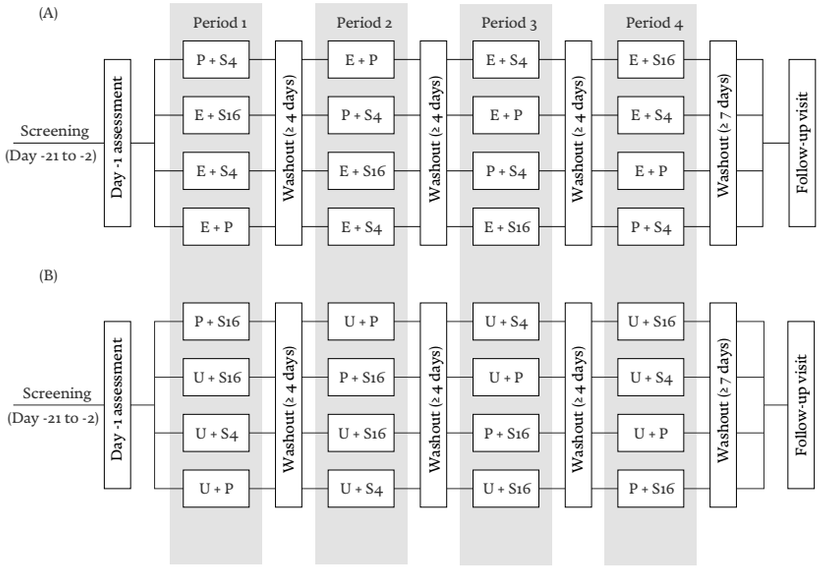
18 S.C. Cannegieter, F.R. Rosendaal, A.R. Wintzen, F.J. van der Meer, *et al., Optimal oral anticoagulant therapy in patients with mechanical heart valves.* N Engl J Med, 1995. 333(1): p. 11-17.

19 E.M. Hylek, S.J. Skates, M.A. Sheehan, and D.E. Singer, *An analysis of the lowest effective intensity of prophylactic anticoagulation for patients with nonrheumatic atrial fibrillation.* N Engl J Med, 1996. 335(8): p. 540-546.

20 H.J. Kleijn, D.P. Zollinger, M.W. van den Heuvel, and T. Kerbusch, *Population pharmacokinetic-pharmacodynamic analysis for sugammadex-mediated reversal of rocuronium-induced neuromuscular blockade.* Br J Clin Pharmacol, 2011. 72(3): p. 415-433.

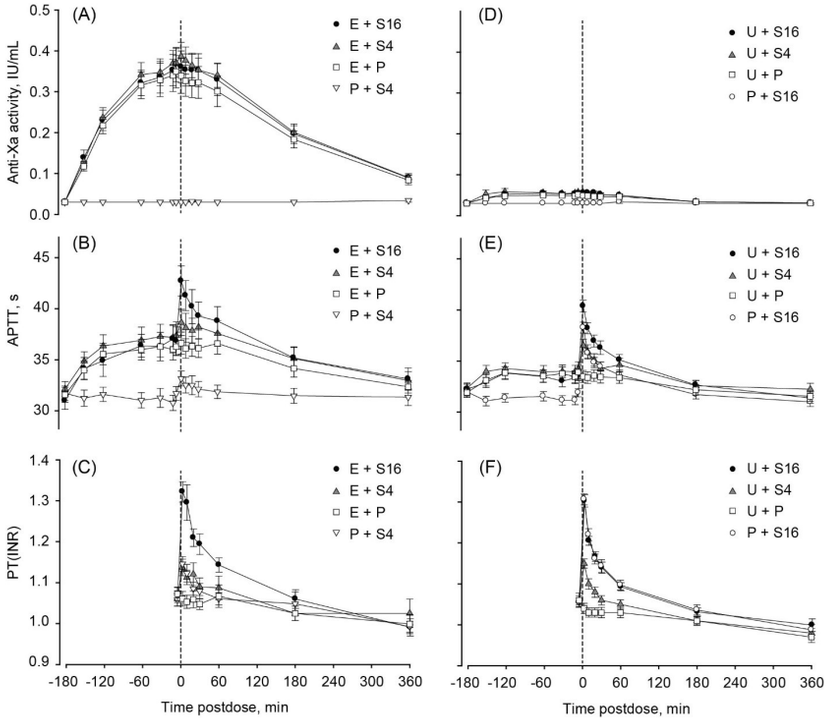
21 L.M. Staals, M.M. Snoeck, J.J. Driessen, H.W. van Hamersvelt, *et al.*, *Reduced clearance of rocuronium and sugammadex in patients with severe to end-stage renal failure: a pharmacokinetic study.* Br J Anaesth, 2010. 104(1): p. 31-39.
22 P.A.M. Peeters, M.W. van den Heuvel, E.V. Heumen, P. Passier, *et al.*, *Safety, tolerability and pharmacokinetics of sugammadex using single high doses (up to 96 mg/kg) in healthy adult subjects: a randomized, double-blind, crossover, placebo-controlled, single-centre study.* Clin Drug Investig, 2010. 30(12): p. 867-874.

Figure 1 The sequences of treatments administered Parts 1 and 2 of this study (A and B, respectively).



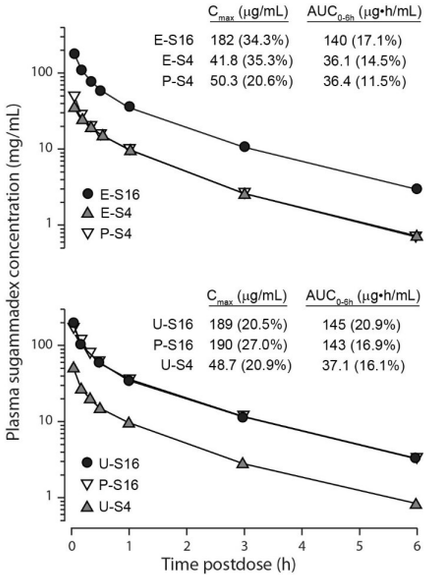
E = enoxaparin 40 mg; P = placebo; S4 and S16 = 4 and 16 mg/kg sugammadex, respectively; and U = unfractionated heparin 5,000 units.

Figure 2 Mean (±SE) anti-Xa activity, activated partial thromboplastin time (APTT), and international normalized ratio for prothrombin time (PT(INR)) over time after treatment with sugammadex or placebo (at time 0) preceded by pretreatment with (A-C) enoxaparin or placebo, or (D-F) unfractionated heparin (UFH) or placebo. Pretreatments were administered immediately after −180 minutes. Blood was not analyzed for PT(INR) prior to −5 minutes.



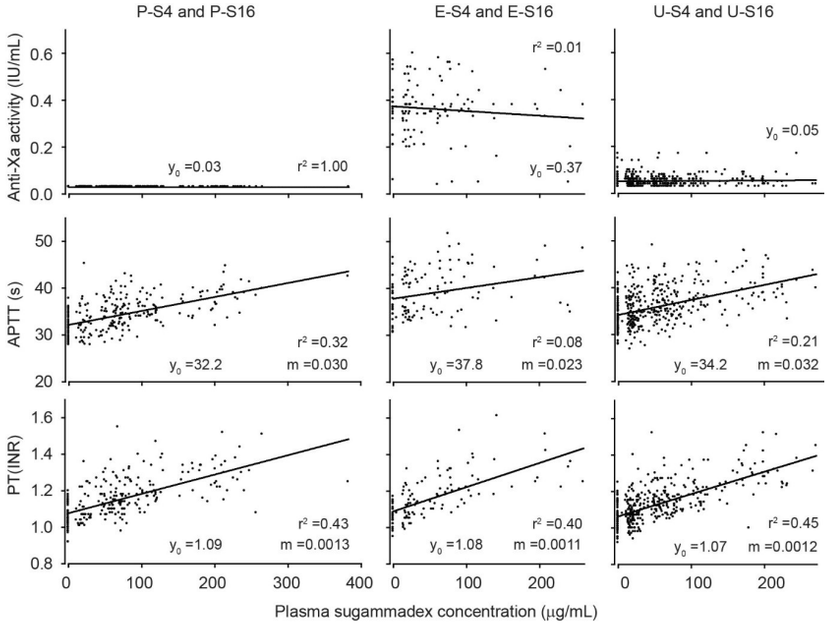
APTT = activated partial thromboplastin time; E = 40 mg enoxaparin; P = placebo; PT(INR) = international normalized ratio for prothrombin time; S4 and S16 = 4 and 16 mg/kg sugammadex, respectively; SE = standard error; U = 5,000 units of UFH; and UFH = unfractionated heparin.

Figure 3 Geometric mean plasma sugammadex concentration over time in subjects administered (upper panel; n = 13) enoxaparin followed by 4 or 16 mg/kg sugammadex (E-S4 and E-S16, respectively) or enoxaparin placebo followed by 4 mg/kg sugammadex (P-S4), or (lower panel; n = 41) unfractionated heparin followed by 4 or 16 mg/kg sugammadex (U-S4 and U-S16) or unfractionated heparin placebo followed by 16 mg/kg sugammadex (U-S16). Values for C_{max} and $AUC_{0-6\ h}$ are geometric means (% geometric coefficient of variation).



$AUC_{0-6\ h}$ = area under the concentration-time curve from 0 to 6 hours; C_{max} = maximum plasma concentration; E = enoxaparin 40 mg; P = placebo; S4 and S16 = 4 and 16 mg/kg sugammadex, respectively; and U = unfractionated heparin 5,000 units.

Figure 4 Anti-Xa activity, activated partial thromboplastin time (APTT), and international normalized ratio for prothrombin time (PT(INR)) *versus* plasma sugammadex concentration after pretreatments with enoxaparin placebo or unfractionated heparin (UFH) placebo (P-S4 and P-S16, left column), enoxaparin (E-S4 and E-S16, middle column), and UFH (U-S4 and U-S16, right column). The analysis included measurements made 5 minutes prior to and at 3, 10, 20, and 30 minutes post-dose to sugammadex. Superimposed lines indicate best fit by linear regression analysis. When not indicated, values for coefficient of determination (R^2) and slope (M) were < 0.01 and < 0.001, respectively.



APTT = activated partial thromboplastin time; E = 40 mg enoxaparin; M = slope; P = placebo; PT(INR) = international normalized ratio for prothrombin time; R^2 = coefficient of determination; S4 and S16 = 4 and 16 mg/kg sugammadex, respectively; U = unfractionated heparin 5,000 units; and Y_0 = y-axis intercept.

Table 1 Baseline demographic characteristics and coagulation status.

	Part 1 (n = 13)	Part 2 (n = 43)
Age, years (mean ±SD)	23.3 ±3.7	24.3 ±5.4
Race (number, %)		
Caucasian	12 (92)	39 (91)
Asian	0	1 (2)
Multiracial	1 (8)	3 (7)
Weight, kg (mean ±SD)	75.4 ±9.0	77.6 ±9.5
Height, cm (mean ±SD)	183.6 ±6.8	183.5 ±6.1
BMI, kg/m² (mean ±SD)	22.6 ±2.4	23.0 ±2.8
Anti-Xa activity, IU/mL (mean ±SD)[a]	0.03[b] ±0.00	0.03[b] ±0.00
APTT, s (mean ±SD)[a]	31.0 ±2.9	32.0 ±2.7
PT(INR) (mean ±SD)[c]	1.06 ±0.6	1.06 ±0.7

(a) Measured in Period 1 prior to dosing with enoxaparin or placebo; (b) all values were < 0.03 IU/mL, the limit of quantitation; and (c) baseline PT(INR) was measured 5 minutes prior to dosing with sugammadex or placebo in subjects who had been pretreated with either the enoxaparin placebo or the unfractionated heparin placebo. APTT = activated partial thromboplastin time; BMI = body mass index; PT(INR) = international normalized ratio for prothrombin time; and SD = standard deviation.

Table 2 Geometric mean values for anti-Xa activity and activated partial thromboplastin time (APTT) time-averaged over the interval from 3 to 30 minutes post-dose to treatments with sugammadex or placebo.

Treatment combination	Geometric mean (95% CI)	
	Anti-Xa activity (IU/mL)	APTT (s)
E + P (n = 12)	0.31 (0.30–0.33)	36.8 (36.0–37.5)
E + S4 (n = 12)	0.32 (0.31–0.33)	37.4 (36.6–38.2)
E + S16 (n = 12)	0.32 (0.31–0.34)	40.2 (39.4–41.0)
U + P (n = 41)	0.05 (0.05–0.05)	33.3 (32.9–33.7)
U + S4 (n = 40)	0.05 (0.05–0.05)	34.8 (34.4–35.2)
U + S16 (n = 39)	0.05 (0.05–0.05)	37.8 (37.3–38.2)

APTT = activated partial thromboplastin time; CI = confidence interval; E = enoxaparin 40 mg; P = placebo; S4 and S16 = sugammadex 4 mg/kg and 16 mg/kg, respectively; and U = unfractionated heparin 5,000 units.

Table 3 Geometric mean ratios (90% confidence intervals) derived by between-treatment comparisons of anti-Xa activity and activated partial thromboplastin time (APTT). Except as noted, the ratios compare mean values that were time-averaged over the interval from 3 to 30 minutes post-dose to treatment with sugammadex or placebo.

Comparison	Anti-Xa activity	APTT
P + S4, post *versus* pre[b]	1.00 (0.90–1.10)	1.05 (1.02–1.08)
P + S16, post *versus* pre[b]	0.97 (0.91–1.04)	1.13 (1.11–1.15)
E + S4 *versus* E + P	1.02 (0.98–1.07)[a]	1.02 (0.99–1.05)
E + S16 *versus* E + P	1.04 (0.99–1.08)[a]	1.09 (1.06–1.13)
E + S4 *versus* P + S4	11.64 (9.96–13.60)	1.19 (1.14–1.24)
U + S4 *versus* U + P	1.00 (0.96–1.03)	1.04 (1.03–1.06)[a]
U + S16 *versus* U + P	1.05 (1.00–1.09)	1.13 (1.12–1.15)[a]
U + S16 *versus* P + S16	1.68 (1.53–1.83)	1.07 (1.05–1.09)

(a) Primary comparisons in which the pre-specified criterion for potential clinical relevance was an upper 90% CI bound > 1.50; and (b) comparison between time-averaged values from 3 to 30 minutes post-dose and –30 to –5 minutes pre-dose to sugammadex or its placebo. APTT = activated partial thromboplastin time; CI = confidence interval; E = enoxaparin 40 mg; P = placebo; S4 and S16 = 4 and 16 mg/kg sugammadex, respectively; and U = unfractionated heparin 5,000 units.

CHAPTER 7

Summary and general discussion

This thesis describes the pharmacological studies into the off-target effect of sugammadex (Bridion®, laboratory code ORG 25969) on coagulation. Sugammadex is a modified γ-cyclodextrin which encapsulates steroidal neuromuscular blocking agents rocuronium and vecuronium and thereby dose-dependently, rapidly and completely reverses their pharmacological effect in the post-operative setting.[1–7] An intravenous dose of 2 and 4 mg/kg sugammadex is recommended for routine reversal of moderate and deep blockade in adults, respectively, and 16 mg/kg sugammadex for reversal 3 minutes after an intubating dose of 1.2 mg/kg rocuronium.[7]

In vitro spiking experiments carried out during the development trajectory of sugammadex showed that 100 µg/mL sugammadex (corresponding with a dose of 8 mg/kg) significantly prolonged activated partial thromboplastin time (APTT) and international normalized ratio for prothrombin time PT(INR),[8] but values remained within normal ranges.[9] The effect of sugammadex on coagulation was not further evaluated in any clinical trial rendering its clinical relevance unknown.[10] This raised safety concerns by the European Medicines Agency (EMA)[11] and the United States Food and Drug Administration (FDA) during their review of the application for marketing authorization for sugammadex.[12] In an effort to address this concern, a post-hoc analysis of all adverse events related to hemorrhage in phase 2/3 trials was performed. Such events occurred in 5.7% and 3.1% of the sugammadex- and placebo-treated subjects, respectively. When the analysis was limited to surgery related bleedings and extended to the total sugammadex group, the incidence decreased to 2.8% for sugammadex subjects and 2.3% for placebo subjects (no statistically significant difference).[11,13] However, the same data resulted in different regulatory decisions in July 2008. While marketing authorization was granted in the European Union,[14] the FDA rejected the application because of deficiencies regarding the characterization of sugammadex effects on coagulation and allergic reactions (the latter mainly concerned the lack of data on safety of repeat exposures).[12] The EMA decided, as risk mitigation, to include the effect of sugammadex on APTT and PT(INR) in the summary of product characteristics (SPC). In addition, dedicated pharmacology studies to investigate the off-target effect of sugammadex on coagulation had to be undertaken as post-authorization commitment.[11] Such studies were also required for resubmission to the FDA.[12] Most of these studies were performed by the Centre for Human Drug Research (CHDR) and collaborators, as described in this thesis. These comprised a variety of *in vitro, ex vivo* and *in vivo* (clinical) pharmacology studies.

For evaluation of the potential clinical relevance of sugammadex induced coagulation effects, understanding the underlying mode of action (MoA) is of importance. In **CHAPTER 2**, a stepwise *in vitro* approach was taken to unravel this MoA. The first step was to scrutinize which component of the drug substance sugammadex is driving the APTT and PT prolongations. During the synthesis of sugammadex (ORG 25969), the related γ-cyclodextrin ORG 48302 is formed that is present up to 7% in the drug substance. ORG 25969 was found to be the major determinant of the sugammadex effects on coagulation parameters and was, therefore, selected for the subsequent experiments. APTT and PT increased with approximately 10 and 2.5 seconds, respectively, at a concentration of 200 µg/mL ORG 25969 which corresponds to the mean peak plasma concentration reached at the dose recommended for immediate reversal in emergency situations of 16 mg/kg. Next, the effect of ORG 25969 on a variety of (adapted) clotting assays addressing coagulation aspects such as thrombin activity, thrombin generation, factor Xa activity and factor Xa generation was explored. These showed that sugammadex is likely to decrease factor Xa activity in the common pathway and activation of factor X specifically in the intrinsic pathway.

The effect of sugammadex on coagulation increases the possibility of interaction with anticoagulant/antiplatelet compounds administered in the perioperative setting and may expose surgical patients to an increased bleeding risk. These potential interactions were first explored in a series of *in vitro* experiments as described in **CHAPTER 3**. Sugammadex (ORG 25969) was added to plasma of patients on a vitamin K antagonist with elevated INRs and to plasma of healthy volunteers spiked with either a low or high level of enoxaparin, fondaparinux, rivaroxaban, or dabigatran. In all conditions, sugammadex induced concentration-dependent increases in APTT and PT(INR), mainly in a proportional manner, with the strongest increases recorded for dabigatran and rivaroxaban. Furthermore, sugammadex demonstrated a similar pattern of APTT and PT(INR) prolongations in perioperatively collected patient plasmas and in control plasma. It was also highlighted that both rocuronium and vecuronium counteract the effect of sugammadex on APTT and PT suggesting that the prolongations are completely neutralized when equimolar concentrations of rocuronium or vecuronium and sugammadex

are present. These findings, combined with the transient nature of sugammadex effects on coagulation and the perioperative management of the investigated compounds, are unlikely to translate into an increased bleeding risk in the perioperative setting, although this possibility cannot be excluded for scenarios not clinically studied.

The potential interactions between sugammadex and thromboprophylactic agents used in the perioperative setting were further evaluated in clinical pharmacology studies. **CHAPTER 4** reports on a feasibility study of using *ex vivo* collagen-induced whole blood platelet aggregometry for evaluation of potential aspirin-drug interactions affecting platelet aggregation in preparation of a sugammadex-aspirin interaction study. Healthy male volunteers received a daily oral dose of 75 mg aspirin for 6 consecutive days. Whole blood platelet aggregation in response to various collagen concentrations was assessed during the day before start of the aspirin treatment and on the last day of treatment. This methodology was found to be robust in terms of assay reproducibility and intra-subject variability. Platelet aggregation was inhibited after aspirin administration and the effect size varied with the collagen concentration. Collagen concentrations of 1 to 2 µg/mL rendered sufficient window to evaluate a potential aspirin-drug interaction on platelet aggregation. These findings were taken into account for the design of the sugammadex-aspirin interaction study in healthy male volunteers as described in **CHAPTER 5**. Subjects randomly received 4 mg/kg sugammadex or placebo intravenously in absence (treatment period 1 and 2) or presence (treatment period 3 and 4) of aspirin. The administration in treatment period 3 and 4 occurred after at least 7 and 11 consecutive days of once daily oral treatment of 75 mg aspirin, respectively, with a maximum of 16 consecutive days of aspirin intake. The pharmacodynamic assessments included whole blood platelet aggregation induced by 1.5 µg/mL collagen, APTT, cutaneous bleeding time, and PT(INR). Aspirin inhibited platelet aggregation and prolonged cutaneous bleeding time, while sugammadex prolonged APTT and PT(INR). No clinically meaningful interaction between sugammadex and aspirin was observed.

In **CHAPTER 6**, the potential interaction between 4 and 16 mg/kg sugammadex and enoxaparin or unfractionated heparin (UFH) on anticoagulant activity in healthy male volunteers was evaluated. Subjects received a subcutaneous abdominal injection of 40 mg enoxaparin (study part 1), 5,000 units of UFH (study part 2) or anticoagulant placebo followed by an intravenous dose of 0, 4 or 16 mg/kg sugammadex 3 hours later. Study part 1 consisted of 4 treatment periods in random order with anticoagulant placebo in combination with 4 mg/kg sugammadex and enoxaparin in combination with 0, 4 or 16 mg/kg sugammadex. Study part 2 consisted of 4 treatment periods in random order with anticoagulant placebo in combination with 16 mg/kg sugammadex and UFH in combination with 0, 4 or 16 mg/kg sugammadex. Anti-Xa activity and APTT were selected as primary endpoints for enoxaparin and UFH, respectively. Other assessments included APTT (for enoxaparin), anti-Xa activity (for UFH), and PT(INR). No clinically relevant effect of sugammadex on enoxaparin or UFH anticoagulant activity was revealed. These findings were further substantiated by exploratory pharmacokinetic/pharmacodynamic (PK/PD) modeling, which showed no effect of sugammadex on anti-Xa activity in presence of enoxaparin, UFH or anticoagulant placebo. Furthermore, similar positive relationships between sugammadex concentration and APTT or PT(INR) were observed, regardless of anticoagulant pretreatment.

In order to evaluate the clinical relevance of the anticoagulant effect of sugammadex, the potential impact of sugammadex on several concomitant thromboprophylactic therapies has been addressed by the sugammadex-drug interaction *in vitro* experiments (**CHAPTER 3**) and studies in healthy volunteers (**CHAPTER 5** and **6**). However, drug-induced anticoagulation is not solely determining bleeding risk in the perioperative setting. Other factors such as nature of the surgery and underlying medical conditions (*e.g.* coagulation factor defects) can contribute as well.[15] Therefore, the research into the clinical relevance of sugammadex effects on coagulation continued in the target patient population. A subpopulation of patients at increased bleeding risk due to intraoperative thromboprophylaxis combined with a major surgical procedure was investigated. This included the effect of reversal of neuromuscular blockade with 4 mg/kg sugammadex *versus* usual care (neostigmine or spontaneous recovery) on bleeding risk in patients undergoing hip or knee joint replacement or hip fracture surgery and receiving commonly prescribed thromboprophylaxis (mainly low molecular weight heparin (LMWH)).[16] Sugammadex induced limited, transient APTT and PT(INR) prolongations, but without increasing the incidence of bleeding or severity of bleeding compared to usual care.

Data of this patient study and the sugammadex-enoxaparin/UFH interaction study (**CHAPTER 6**) were used to build PK-APTT and PK-PT(INR) models

for prediction of the anticoagulant effects of sugammadex in the patient population in scenarios not clinically evaluated, such as treatment with 16 mg/kg sugammadex.[17] This dose is rarely used, only in case of rescue reversal where patients are in a potentially life-threatening situation and restoring the airway is the immediate concern[7,18] which clearly outweighs the potential increased bleeding risk associated with sugammadex. Additionally, surgery has generally not been initiated at such stage and is likely to be postponed if the surgery is not urgent. Nonetheless, modeling the APTT and PT(INR) effects upon treatment with 16 mg/kg sugammadex complements the insights on the effects of sugammadex on coagulation. The relationship between sugammadex plasma concentration and anticoagulant activity was in both APTT and PT(INR) models best described by a maximum effect (E_{max}) function, however, the majority of the data fell below the estimated concentration of sugammadex producing the half-maximal response (EC_{50}), indicating that there were limited data to accurately estimate the potentially maximally achievable effect. Nevertheless, these models predict APTT and PT(INR) increases in surgical patients on thromboprophylaxis receiving 16 mg/kg sugammadex well below the threshold considered to be the minimum clinically relevant meaningful effect of anticoagulant treatment.[17] Data of the sugammadex-aspirin study (**CHAPTER 5**) were used for external validation of these models.

Evaluation of the off-target effects of sugammadex on coagulation as described in this thesis were indispensable to overcome the bleeding safety concerns raised by both the EMA and the FDA. FDA approval was granted on 15 December 2015[19] and reflection on this odyssey of 7.5 years may provide an opportunity to learn from the development trajectory of sugammadex. At CHDR, we advocate the so-called question-based drug development approach.[20–22] During a clinical drug development program, a number of generic questions need to be answered on the pharmacology of a drug. These questions range from the compound's absorption, distribution, metabolism and excretion (does the compound get to the site of action?) to the sources of variability in drug response in the target population. These questions are used to design clinical trials and their objectives rather than the traditional successive 4 clinical phases approach. Answering all questions reduces the uncertainty about a drug and thereby minimizes its developmental risk. Question-based drug development addresses both the on-target and off-target pharmacology of a drug; the latter requires for instance knowledge on the etiology of an off-target effect. This knowledge on the *in vitro* effects of sugammadex on APTT and PT(INR) was missing in the original application of sugammadex, which prompted the regulators to require additional studies dedicated to establish the clinical relevance of the anticoagulant effect of sugammadex. An interesting question is what would have happened when the development trajectory had included detailed coagulation assessments in the early clinical studies. It is tempting to speculate that earlier availability of such data would have contributed to earlier clinical acceptance of sugammadex by the regulatory authorities.

In conclusion, this thesis shows that when sugammadex and anticoagulants are administered according to their labels in the perioperative setting, the bleeding risk can be considered negligible. However, off-label use such as in patients with (high) clinically relevant anticoagulant levels in an emergency surgery situation should be cautiously handled. Asking and answering all relevant scientific questions related to off-target effects during the early clinical development phase may have unlocked the true potential of sugammadex earlier.

REFERENCES

1 A. Abrishami, J. Ho, J. Wong, L. Yin, *et al.*, *Cochrane corner: sugammadex, a selective reversal medication for preventing postoperative residual neuromuscular blockade.* Anesth Analg, 2010. 110(4): p. 1239.

2 M. Carron, F. Zarantonello, P. Tellaroli, and C. Ori, *Efficacy and safety of sugammadex compared to neostigmine for reversal of neuromuscular blockade: a meta-analysis of randomized controlled trials.* J Clin Anesth, 2016. 35: p. 1-12.

3 W.T. Nicholson, J. Sprung, and C.J. Jankowski, *Sugammadex: a novel agent for the reversal of neuromuscular blockade.* Pharmacotherapy, 2007. 27(8): p. 1181-1188.

4 S.J. Schaller and H. Lewald, *Clinical pharmacology and efficacy of sugammadex in the reversal of neuromuscular blockade.* Expert Opin Drug Metab Toxicol, 2016. 12(9): p. 1097-1108.

5 A. Srivastava and J.M. Hunter, *Reversal of neuromuscular block.* Br J Anaesth, 2009. 103(1): p. 115-129.

6 M. Welliver, *New drug sugammadex: a selective relaxant binding agent.* AANA J, 2006. 74(5): p. 357-363.

7 W.J. Herring, T. Woo, C.A. Assaid, R.J. Lupinacci, *et al.*, *Sugammadex efficacy for reversal of rocuronium- and vecuronium-induced neuromuscular blockade: a pooled analysis of 26 studies.* J Clin Anesth, 2017. 41: p. 84-91.

8 P.J. de Kam, P. Grobara, M. Prohn, F. Höppener, *et al.*, *Effects of sugammadex on activated partial thromboplastin time and prothrombin time in healthy subjects.* Int J Clin Pharmacol Ther, 2014. 52(3): p. 227-236.

9 Food and Drug Administration Center for Drug Evaluation and Research. *Application Number: 022225Orig1s000, Clinical Pharmacology and Biopharmaceutics Review(s).* 2015; Available from: https://www.accessdata.fda.gov/drugsatfda_docs/nda/2015/022225Orig1s000ClinPharmR.pdf.

10 Food and Drug Administration Center for Drug Evaluation and Research. *Application Number: 022225Orig1s000, Other Review(s).* 2015; Available from: https://www.accessdata.fda.gov/drugsatfda_docs/nda/2015/022225Orig1s000OtherR.pdf.

11 European Medicines Agency. *Assessment Report for Bridion*. 2008; Available from: www.ema.europa.eu/docs/en_GB/document_library/EPAR_-_Public_assessment_report/human/000885/WC500052309.pdf.

12 Food and Drug Administration Center for Drug Evaluation and Research. *Application Number: 022225Orig1s000, Other Action Letters*. 2015; Available from: https://www.accessdata.fda.gov/drugsatfda_docs/nda/2015/022225Orig1s000OtherActionLtr.pdf.

13 Food and Drug Administration Center for Drug Evaluation and Research. *Application Number: 022225Orig1s000, Medical Review(s)*. 2015; Available from: https://www.accessdata.fda.gov/drugsatfda_docs/nda/2015/022225Orig1s000MedR.pdf.

14 European Union. *Official Journal of the European Union, Information and Notices; Volume 51, 29 August 2008*. 2008; Available from: http://eur-lex.europa.eu/legal-content/EN/TXT/HTML/?uri=OJ:C:2008:220:FULL&from=EN.

15 M.B. Koh and B.J. Hunt, *The management of perioperative bleeding.* Blood Rev, 2003. 17(3): p. 179-185.

16 N. Rahe-Meyer, H. Fennema, S. Schulman, W. Klimscha, *et al.*, *Effect of reversal of neuromuscular blockade with sugammadex versus usual care on bleeding risk in a randomized study of surgical patients.* Anesthesiology, 2014. 121(5): p. 969-977.

17 R. Bosch, M.J. van Lierop, P.J. de Kam, A.C. Kruithof, *et al.*, *A PK-PD model-based assessment of sugammadex effects on coagulation parameters.* Eur J Pharm Sci, 2016. 84: p. 9-17.

18 C. Frerk, V.S. Mitchell, A.F. McNarry, C. Mendonca, *et al.*, *Difficult Airway Society 2015 guidelines for management of unanticipated difficult intubation in adults.* Br J Anaesth, 2015. 115(6): p. 827-848.

19 Food and Drug Administration Center for Drug Evaluation and Research. *Approval Package for Application Number: 022225Orig1s000*. 2015; Available from: https://www.accessdata.fda.gov/drugsatfda_docs/nda/2015/022225Orig1s000Approv.pdf.

20 A.F. Cohen, *Developing drug prototypes: pharmacology replaces safety and tolerability?* Nat Rev Drug Discov, 2010. 9(11): p. 856-865.

21 A.F. Cohen, J. Burggraaf, J.M. van Gerven, M. Moerland, *et al.*, *The use of biomarkers in human pharmacology (Phase I) studies.* Annu Rev Pharmacol Toxicol, 2015. 55: p. 55-74.

22 S.J. de Visser, *A question based approach to drug development*. 2003, Leiden University, Doctoral thesis.

CHAPTER 8

Summary in Dutch

In dit proefschrift worden de farmacologische experimenten naar de ongewenste effecten van sugammadex (Bridion®, laboratorium code ORG 25969) op coagulatie beschreven. Sugammadex is een gemodificeerde γ-cyclodextrine die de steroïde neuromusculair blokkerende stoffen rocuronium en vecuronium kan inkapselen. Hierdoor heft sugammadex dosisafhankelijk, snel en volledig de spierverslappende werking op van deze spierrelaxantia die worden gegeven als onderdeel van anesthesie bij chirurgische ingrepen. Sugammadex wordt intraveneus toegediend. De aanbevolen dosis is 2 en 4 mg/kg voor standaardopheffing van een respectievelijk gematigde en diepe neuromusculaire blokkade bij volwassenen. Voor onmiddellijke opheffing is een dosis van 16 mg/kg sugammadex 3 minuten na toediening van 1,2 mg/kg rocuronium aanbevolen.

Tijdens het ontwikkelingstraject van sugammadex werd bij experimenten in het laboratorium (*in vitro*) sugammadex toegevoegd aan plasmamonsters. Vervolgens werden de geactiveerde partiële tromboplastinetijd (APTT) en prothrombinetijd (PT) uitgedrukt in de internationaal genormaliseerde ratio (INR) bepaald. Na toevoeging van 100 µg/mL sugammadex, wat overeenkomt met een dosis van 8 mg/kg, waren de APTT en PT(INR) significant verlengd, maar binnen de normaalwaarden. Het effect van sugammadex op coagulatie werd niet verder onderzocht in een klinisch onderzoek waardoor de klinische relevantie ervan onbekend bleef. Dit leidde tot zorgen bij het Europees Geneesmiddelenbureau (European Medicines Agency, EMA) en de geneesmiddelen-toezichthouder van de Verenigde Staten van Amerika (Food and Drug Administration, FDA) tijdens de beoordeling van de registratieaanvraag voor sugammadex. In een poging om deze zorgen weg te nemen werd een post-hoc analyse van alle bloeding-gerelateerde bijwerkingen in fase 2/3-studies uitgevoerd. De incidentie van deze bijwerkingen was 5,7% in de sugammadex-groep en 3,1% in de placebo-groep. Wanneer de analyse werd uitgevoerd op basis van alleen de bloedingen die gerelateerd waren aan chirurgische ingrepen in de totale sugammadex-groep, nam de incidentie af naar 2,8% voor sugammadex en 2,3% voor placebo (geen statistisch significant verschil). Dit leidde echter tot verschillende besluiten van de registratieautoriteiten in juli 2008. In de Europese Unie (EU) werd sugammadex goedgekeurd terwijl de FDA geen goedkeuring verleende vanwege hiaten in de registratieaanvraag met betrekking tot karakterisering van de effecten van sugammadex op coagulatie en allergische reacties (dat laatste betrof met name het gebrek aan data over herhaalde blootstelling aan sugammadex). De EMA besloot het effect van sugammadex op APTT en PT(INR) in de samenvatting van productkenmerken (SPC) te vermelden als risicobeperking. Verder werd er toegezegd dat farmacologische experimenten naar het ongewenste effect van sugammadex op coagulatie onderdeel zouden zijn van de vervolgonderzoeken die na registratie moesten worden uitgevoerd. Dergelijke experimenten waren ook nodig voor de herindiening bij de FDA. De meeste van deze onderzoeken zijn uitgevoerd door het Centre for Human Drug Research (CHDR) en partners, en worden beschreven in dit proefschrift. Deze bestonden uit verschillende farmacologische experimenten met menselijk materiaal (*in vitro* en *ex vivo*) en klinische onderzoeken (*in vivo*).

Om de mogelijke klinische relevantie van het effect van sugammadex op coagulatie te kunnen bepalen is kennis van het onderliggende mechanisme van belang. In **HOOFDSTUK 2** wordt dit mechanisme stapsgewijs *in vitro* onderzocht. De eerste stap was het bepalen welke component van het werkzame bestanddeel van sugammadex de APTT- en PT-verlengingen veroorzaakt. Tijdens de synthese van sugammadex (ORG 25969) wordt de gerelateerde γ-cyclodextrine ORG 48302 gevormd wat tot 7% aanwezig is in het werkzame bestanddeel. ORG 25969 bleek de belangrijkste determinant van het effect van sugammadex op coagulatieparameters te zijn, en werd daarom geselecteerd voor de vervolgexperimenten. APTT en PT waren verlengd met respectievelijk 10 en 2,5 seconde bij een concentratie van 200 µg/mL ORG 25969, wat overeenkomt met de gemiddelde maximale plasmaconcentratie na een dosis voor onmiddellijke opheffing in noodsituaties van 16 mg/kg. Vervolgens werd het effect van ORG 25969 op diverse (aangepaste) coagulatietesten zoals trombine-activiteit, trombinegeneratie, factor Xa-activiteit en factor Xa-generatie onderzocht. Deze toonden aan dat sugammadex waarschijnlijk factor Xa-activiteit in de gemeenschappelijke route en factor Xa-activatie specifiek in de intrinsieke route remt.

Het effect van sugammadex op coagulatie vergroot de kans op interactie met anticoagulantia/plaatjesaggregatieremmers die worden toegediend in de perioperatieve setting. Dit zou kunnen zorgen voor een verhoogd bloedingsrisico bij chirurgische patiënten. Deze mogelijke interacties werden eerst onderzocht in een serie *in vitro* experimenten zoals beschreven in **HOOFDSTUK 3**. Sugammadex (ORG 25969) werd toegevoegd aan plasma van patiënten met verhoogde INRs en behandeld met vitamine K-antagonisten, en aan plasma van gezonde vrijwilligers verrijkt met een lage of hoge concentratie enoxaparine, fondaparinux, rivaroxaban of dabigatran. Bij alle condities induceerde

sugammadex concentratie-afhankelijke verlengingen van APTT en PT(INR), overwegend proportioneel, met de sterkste verlengingen bij dabigatran en rivaroxaban. Verder waren de APTT- en PT(INR)-verlengingen na toevoeging van sugammadex aan plasma van patiënten in de perioperatieve setting vergelijkbaar met controleplasma. Ook werd aangetoond dat zowel rocuronium als vecuronium het effect van sugammadex op APTT en PT tegengaat wat suggereert dat de verlengingen volkomen geneutraliseerd worden als de concentraties van rocuronium of vecuronium en sugammadex equimolair zijn. Deze bevindingen, in combinatie met de tijdelijke aard van sugammadexeffecten op coagulatie en het perioperatieve beleid van de onderzochte geneesmiddelen, maken het niet aannemelijk dat sugammadex-behandeling zich vertaald naar een verhoogd bloedingsrisico in de perioperatieve setting. Echter kan dit niet worden uitgesloten voor scenario's die niet klinisch zijn onderzocht. De mogelijke interacties van sugammadex met tromboprofylactische middelen die worden gebruikt in de perioperatieve setting werd verder bestudeerd in klinisch farmacologische studies. **HOOFDSTUK 4** rapporteert een haalbaarheidsstudie naar het gebruik van *ex vivo* collageen-geïnduceerde volbloed plaatjesaggregatie voor de evaluatie van mogelijke aspirine-geneesmiddelinteracties op plaatjesaggregatie ter voorbereiding van een sugammadex-aspirine interactiestudie. Gezonde mannelijke vrijwilligers namen dagelijks oraal 75 mg aspirine in gedurende een periode van 6 dagen. Plaatjesaggregatie werd na toevoeging van verschillende concentraties collageen bepaald gedurende de dag voor aanvang van aspirine behandeling en op de laatste dag van behandeling. Deze methodologie bleek robuust te zijn in termen van reproduceerbaarheid en intra-subject variabiliteit. Plaatjesaggregatie was geremd na aspirine inname en de mate van remming was afhankelijk van de collageenconcentratie. Bij collageenconcentraties van 1 tot 2 µg/mL was er nog voldoende ruimte om een mogelijke aspirine-geneesmiddelinteractie op plaatjesaggregatie te detecteren. Deze bevindingen werden meegenomen bij het ontwerp van de sugammadex-aspirine interactiestudie in gezonde mannelijke vrijwilligers beschreven in **HOOFDSTUK 5**. Vrijwilligers kregen in willekeurige volgorde 4 mg/kg sugammadex of placebo intraveneus toegediend zonder (behandelingsperiode 1 en 2) of met (behandelingsperiode 3 en 4) aspirine. De toediening in behandelingsperiode 3 en 4 vond plaats na respectievelijk tenminste 7 en 11 opeenvolgende dagen van eenmaal daags inname van 75 mg aspirine met maximaal 16 opeenvolgende dagen van aspirine inname. De farmacodynamische bepalingen waren volbloed plaatjesaggregatie (geïnduceerd door 1,5 µg/mL collageen), APTT, bloedingstijd en PT(INR). Aspirine

remde plaatjesaggregatie en verlengde de bloedingstijd terwijl sugammadex APTT en PT(INR) verlengde. Er werd geen klinische betekenisvolle interactie tussen sugammadex en aspirine gevonden.

In **HOOFDSTUK 6** werd de mogelijke interactie tussen 4 en 16 mg/kg sugammadex en enoxaparine of ongefractioneerde heparine op antistollingsactiviteit in gezonde mannelijke vrijwilligers onderzocht. Vrijwilligers kregen een subcutane injectie in de buik van 40 mg enoxaparine (studiedeel 1), 5.000 eenheden van ongefractioneerde heparine (studiedeel 2) of anticoagulans placebo gevolgd door een intraveneuze dosis van 0, 4 of 16 mg/kg sugammadex 3 uur later. Studiedeel 1 bestond uit 4 behandelingsperiodes met in willekeurige volgorde anticoagulans placebo in combinatie met 4 mg/kg sugammadex of enoxaparine in combinatie met 0, 4 of 16 mg/kg sugammadex. Studiedeel 2 bestond uit 4 behandelingsperiodes met in willekeurige volgorde anticoagulans placebo in combinatie met 16 mg/kg sugammadex of ongefractioneerde heparine in combinatie met 0, 4 of 16 mg/kg sugammadex. Anti-Xa-activiteit en APTT waren geselecteerd als belangrijkste uitkomstmaten voor respectievelijk enoxaparine en ongefractioneerde heparine. Overige bepalingen bestonden uit APTT (voor enoxaparine), anti-Xa-activiteit (voor ongefractioneerde heparine) en PT(INR). Er werd geen klinisch relevant effect van sugammadex op de antistollingsactiviteit van enoxaparine of ongefractioneerde heparine vastgesteld. Deze bevindingen werden verder geduid met exploratieve farmacokinetische/farmacodynamische (PK/PD) modellering wat geen effect van sugammadex op anti-Xa-activiteit liet zien in de aanwezigheid van enoxaparine, ongefractioneerde heparine of anticoagulans placebo. Daarnaast werden vergelijkbare correlaties tussen sugammadexconcentratie en APTT of PT(INR) gezien, ongeacht de anticoagulans voorbehandeling.

Om de klinische relevantie van het antistollingseffect van sugammadex te kunnen bepalen werd de mogelijke impact van sugammadex op verschillende tromboprofylactische comedicaties bestudeerd in sugammadex-geneesmiddel interactie-experimenten *in vitro* (**HOOFDSTUK 3**) en in experimenten in gezonde vrijwilligers (**HOOFDSTUK 5** en **6**). Echter wordt het bloedingsrisico in de perioperatieve setting niet alleen bepaald door geneesmiddel-geïnduceerde antistolling. Andere factoren zoals de aard van de chirurgische ingreep en onderliggende medische condities (bijvoorbeeld coagulatiefactor-defecten) kunnen van invloed zijn. Daarom werd het onderzoek naar de klinische relevantie van sugammadex-effecten op coagulatie voortgezet in de patiënten-doelgroep. Een subgroep van patiënten met verhoogd bloedingsrisico ten gevolge van in-

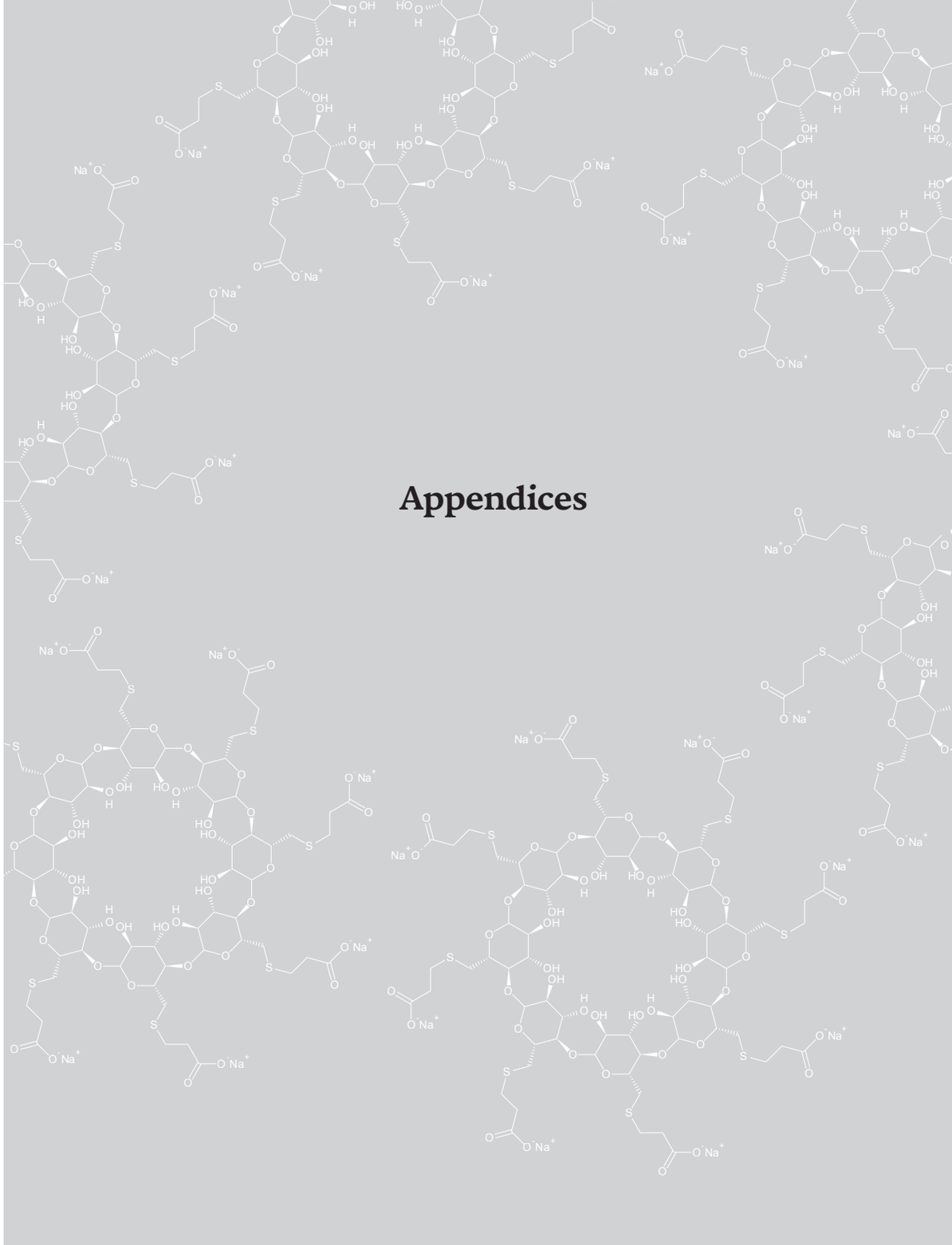
tra-operatieve tromboseprofylaxe in combinatie met een grote chirurgische ingreep werd onderzocht. Dit omvatte het effect van standaardopheffing van een neuromusculaire blok met 4 mg/kg sugammadex *versus* standaardbehandeling (neostigmine of spontaan herstel) op het bloedingsrisico bij patiënten die heup/kniegewrichtsvervanging of heupfractuur-chirurgie ondergingen en behandeld werden met een veel voorgeschreven tromboseprofylaxe (met name laagmoleculair gewicht heparine (LMWH)). Sugammadex zorgde voor geringe, tijdelijke APTT en PT(INR) verlengingen, maar zonder toename van de incidentie of ernst van bloedingen in vergelijking met standaardbehandeling.

Data van deze patiëntenstudie en de sugammadex-enoxaparine/ongefractioneerde heparine interactiestudie (**HOOFDSTUK 6**) werden gebruikt om PK-APTT- en PK-PT(INR)-modellen te maken om de antistollingseffecten van sugammadex in scenario's die niet klinisch onderzocht waren in patiënten-doelgroepen te voorspellen, zoals de behandeling met 16 mg/kg sugammadex. Deze dosis wordt zelden toegepast, alleen bij patiënten die zich in een mogelijk levensbedreigende situatie bevinden waarbij herstel van de luchtwegen onmiddellijk noodzakelijk is. In een dergelijke situatie weegt het gebruik van sugammadex duidelijk zwaarder dan het mogelijke bloedingsrisico geassocieerd met sugammadex. Verder is over het algemeen de operatie nog niet begonnen en zal deze worden uitgesteld indien de operatie niet urgent is. Niettemin zorgt het modelleren van de APTT en PT(INR) effecten na behandeling met 16 mg/kg sugammadex voor aanvullende inzichten in de effecten van sugammadex op coagulatie. De correlatie tussen sugammadex plasmaconcentratie en antistollingsactiviteit werd in zowel het APTT- als PT(INR)-model het beste beschreven door een maximale effect (E_{max})-functie, echter viel het merendeel van de data onder de geschatte sugammadexconcentratie benodigd voor een half maximale response (EC_{50}), wat duidt dat er beperkte data was voor een accurate schatting van het mogelijk maximale effect. Desondanks voorspellen deze modellen dat APTT- en PT(INR)-verlengingen bij chirurgische patiënten die behandeld worden met tromboseprofylaxe en 16 mg/kg sugammadex krijgen toegediend ruim onder de drempelwaarden lagen die minimaal klinisch relevant worden geacht voor antistollingsbehandeling. Data van de sugammadex-aspirine interactiestudie (**HOOFDSTUK 5**) werd gebruikt voor externe validatie van deze modellen.

De evaluatie van de ongewenste effect van sugammadex op coagulatie zoals beschreven in dit proefschrift was essentieel om de zorgen van de EMA en de FDA over de veiligheid met betrekking tot bloedingen te ondervangen. De FDA verleende goedkeuring op 15 december 2015 en reflectie op de 7,5 jaar durende odyssee om deze goedkeuring te verkrijgen kan een mogelijkheid bieden om te leren van het ontwikkelingstraject van sugammadex. Op het CHDR zijn we voorstander van de zogenoemde vraag-gestuurde geneesmiddelenontwikkeling ('question-based drug development'). Gedurende het klinisch geneesmiddelontwikkelingstraject dienen een aantal generieke vragen over de farmacologie van een middel beantwoord te worden. Deze vragen variëren van de absorptie, distributie, metabolisme en excretie van een middel (bereikt het middel de plaats van werking?) tot de variabiliteit van het klinisch effect van een geneesmiddel in doelpopulatie. Deze vragen worden gebruikt voor het ontwerp van klinische onderzoeken en bijbehorende doelstellingen in plaats van de traditionele aanpak van 4 opeenvolgende klinische fases. Het beantwoorden van al deze vragen vermindert de onzekerheid omtrent een geneesmiddel waardoor het ontwikkelingsrisico geminimaliseerd wordt. Bij question-based drug development gaat het om zowel de gewenste als de ongewenste farmacologie van een geneesmiddel, waarbij voor laatstgenoemde bijvoorbeeld kennis van de etiologie van een ongewenst effect vereist is. Deze kennis over de *in vitro* effecten van sugammadex op APTT en PT(INR) ontbrak in het oorspronkelijke aanvraag van sugammadex wat aanleiding was voor de registratieautoriteiten om genoemde aanvullende onderzoeken te eisen om de klinische relevantie van het antistollingseffect van sugammadex vast te stellen.

Een interessante vraag is wat er gebeurd zou zijn als gedetailleerde coagulatiebepalingen waren meegenomen in de eerste klinische onderzoeken. Het is verleidelijk om te speculeren dat eerdere beschikbaarheid van zulke data had bijgedragen aan de klinische acceptatie van sugammadex door registratieautoriteiten.

Concluderend laat dit proefschrift zien dat wanneer sugammadex en anticoagulantia worden toegediend in de perioperatieve setting, in overeenstemming met hun bijsluiters, het bloedingsrisico als verwaarloosbaar kan worden beschouwd. Echter, bij off-label gebruik zoals bij patiënten met (hoge) klinisch relevante concentraties anticoagulans, en die met spoed geopereerd moeten worden, is voorzichtigheid geboden. Door het stellen en beantwoorden van alle relevante wetenschappelijke vragen gerelateerd aan ongewenste effecten in een eerder stadium, tijdens het vroeg-klinische ontwikkelingstraject, had sugammadex' ware potentieel eerder kunnen worden ontgrendeld.



Appendices

CURRICULUM VITAE

Annelieke Charissa Kruithof was born on the 15th of March 1985 in Steenwijk, The Netherlands. After graduation from the secondary school CSG Dingstede in Meppel in 2003, she studied Bio-Pharmaceutical Sciences (BPS) at Leiden University. She also became a member of the Leidsche Pharmaceutische Studenten-Vereeniging (L.P.S.V.) „Aesculapius" and joined several committees including the dies committee. Following her bachelor's degree in 2006, she served as quaestrix of the 121st board of the study association. She continued her education with the BPS master research track Drug Delivery Technology and Bio-Pharmaceutics which she completed *cum laude* in 2010. As part of the master's program, she performed an internship at the dermatology department of the Charité University Hospital in Berlin, Germany. She evaluated three types of microneedle arrays in the skin using *in vivo* laser scanning microscopy in human subjects. This sparked her interest in clinical research and prompted her to start working as Clinical Scientist at the Centre for Human Drug Research (CHDR) in Leiden in 2011. There she began her PhD trajectory as described in this thesis under supervision of dr. M. Moerland and prof. dr. J. Burggraaf. The coagulation part was performed in collaboration with Good Biomarker Sciences, headed by prof. dr. C. Kluft, in Leiden. In addition to her PhD studies, she contributed to numerous other clinical studies across several therapeutic areas including internal medicine and neurology. Whilst working at CHDR, she became a board-certified Clinical Pharmacologist in 2015. Since 2017, she works as Experienced Clinical Scientist at CHDR and is currently member of the Infectious Diseases group.

LIST OF PUBLICATIONS

G.V.T. Roozen, M.L.M. Prins, R.S. van Binnendijk, G. den Hartog, V.P. Kuiper, C. Prins, J.J. Janse, **A.C. Kruithof**, M.C.W. Feltkamp, M. Kuijer, F.R. Roosendaal, M. Roestenberg, L. Visser and A. Roukens, *Tolerability, safety and immunogenicity of intradermal delivery of a fractional dose mRNA-1273 SARS-CoV-2 vaccine in healthy adults as a dose sparing strategy.* Social Science Research Network. Available at: http://dx.doi.org/10.2139/ssrn.3892129.

J.M. den Heijer, J.A.A.C. Heuberger, H. Hijma, **A.C. Kruithof**, J. van Smeden, G.J. Groeneveld, J. Burggraaf and Adam Cohen, *Good Clinical Trials by removing defensive interpretation of Good Clinical Practice guidelines.* British Journal of Clinical Pharmacology, 2021, p. 1-8. Available at: https://doi.org/10.1111/bcp.14843.

J.M. den Heijer, **A.C. Kruithof**, G. van Amerongen, M.L. de Kam, E. Thijssen, H.W. Grievink, M. Moerland, M. Walker, K. Been, R. Skerlj, C. Justman, L. Dudgeon, P. Lansbury, V.C. Cullen, D.C. Hilt and G.J. Groeneveld, *A randomized single and multiple ascending dose study in healthy volunteers of LTI-291, a centrally penetrant glucocerebrosidase activator.* British Journal of Clinical Pharmacology, 2021. 87(9): p. 3561-3573.

A.C. Kruithof, C. Kluft, P.-J. de Kam, G.H. Laterveer, M. Moerland and J. Burggraaf, *The mode of action of sugammadex on coagulation.* International Journal of Clinical Pharmacology and Therapeutics, 2021. 59(2): p. 89-98

A.C. Kruithof, C. Kluft, P.-J. de Kam, G.H. Laterveer, M. Moerland and J. Burggraaf, *Interactions of sugammadex with various anticoagulants.* International Journal of Clinical Pharmacology and Therapeutics, 2020. 58(7): p. 395-403.

S.C. Goulooze, **A.C. Kruithof**, S. Alikunju, A. Gautam, J. Burggraaf, I.M.C. Kamerling and J. Stevens, *The effect of food and formulation on the population pharmacokinetics of cholesteryl ester transferase protein inhibitor DRL-17822 in healthy male volunteers.* British Journal of Clinical Pharmacology, 2020. 86(10): p. 2095-2101.

A.C. Kruithof, R. Kumar, J. Stevens, M.L. de Kam, A. Gautam, S. Alikunju, B.K. Padhi, S. Kulkarni, R.S. Raghuvanshi, R. Gandhi, J. Burggraaf and I.M.C. Kamerling, *Effect of food on the pharmacokinetics of 2 formulations of DRL-17822, a novel selective cholesteryl ester transfer protein (CETP) inhibitor, in healthy males.* Clinical Pharmacology in Drug Development, 2019. 8(8): p. 1042-1052.

A.C. Kruithof, S. Watanabe, P.A.M. Peeters, M.L. de Kam, R.G.J.A. Zuiker, J. Stevens, J.M.A. van Gerven and A. Stockis, *Pharmacological interactions between brivaracetam and ethanol in healthy males.* Journal of Psychopharmacology, 2017. 31(7): p. 915-926.

R. Bosch, M.-J. van Lierop, P.-J. de Kam, **A.C. Kruithof**, J. Burggraaf, R. de Greef, S.A.G. Visser, A.O. Johnson-Levonas and H.-J. Kleijn, *A PK-PD model-based assessment of sugammadex effects on coagulation parameters.* European Journal of Pharmaceutical Sciences, 2016. 84: p. 9-17.

A.C. Kruithof, M. Moerland, E.A. Anastasopoulou, P.-J. de Kam, M.L. de Kam and J. Burggraaf, *Evaluation of the effect of aspirin on platelet aggregation: methodological recommendations for aspirin-drug interaction studies.* Journal of Biomedical Science and Engineering, 2015. 8(1): p. 40-45.

P.-J. de Kam, **A.C. Kruithof**, M.-J. van Lierop, M. Moerland, J. Dennie, M.D. Troyer, R.B. Langdon, D.E. Gutstein, J. Burggraaf and R. El Galta, *Lack of a clinically relevant effect of sugammadex on anti-Xa activity or activated partial thromboplastin time following pretreatment with either unfractionated or low-molecular-weight heparin in healthy subjects.* International Journal of Clinical Pharmacology and Therapeutics, 2014. 52(8): p. 631-641.

P.-J. de Kam, R. El Galta, **A.C. Kruithof**, H. Fennema, M.-J. van Lierop, K. Mihara, J. Burggraaf, M. Moerland, P. Peeters and M.D. Troyer, *No clinically relevant interaction between sugammadex and aspirin on platelet aggregation and coagulation parameters.* International Journal of Clinical Pharmacology and Therapeutics, 2013. 51(12): p. 976-985.

M.C. Meinke, **A.C. Kruithof**, S.M. Bal, J.A. Bouwstra and J. Lademann, *Application of in vivo laser scanning microscopy to visualise the penetration of a fluorescent dye in solution and liposomes into the skin after pretreatment with microneedles.* Laser Scanning, Theory and Applications, 2011. ISBN 978-953-307-205-0: p. 171-184.

S.M. Bal, **A.C. Kruithof**, R. Zwier, E. Dietz, J.A. Bouwstra, J. Lademann and M.C. Meinke, *Influence of microneedle shape on the transport of a fluorescent dye into human skin in vivo.* Journal of Controlled Release, 2010. 147(2): p. 218-224.

A.C. Kruithof*, S. Bal*, H. Liebl, M. Tomerius, J. Bouwstra, J. Lademann and M. Meinke, *In vivo visualization of microneedle conduits in human skin using laser scanning microscopy.* Laser Physics Letter, 2010. 7(3): p. 242-246.

S.M. Bal, B. Slütter, E. van Riet, **A.C. Kruithof**, Z. Ding, G.F.A. Kersten, W. Jiskoot and J.A. Bouwstra, *Efficient induction of immune responses through intradermal vaccination with N-trimethyl chitosan containing antigen formulations.* Journal of Controlled Release, 2010. 142(3): p. 374-383.

** Contributed equally as first authors.*

AUTHOR AFFILIATIONS AT THE TIME OF THE RESEARCH

CENTRE FOR HUMAN DRUG RESEARCH (CHDR), LEIDEN, THE NETHERLANDS

E.A. Anastasopoulou
J. Burggraaf
M.L. de Kam
A.C. Kruithof
M. Moerland

GOOD BIOMARKER SCIENCES (GBS), LEIDEN, THE NETHERLANDS

C. Kluft
R.H. Laterveer

LEIDEN UNIVERSITY MEDICAL CENTER (LUMC), LEIDEN, THE NETHERLANDS

J. Burggraaf
A.C. Kruithof
M. Moerland

MERCK SHARP & DOHME CORP. (MSD), OSS, THE NETHERLANDS

H. Fennema
R. El Galta
M.-J. van Lierop
K. Mihara
P.A.M. Peeters

MERCK SHARP & DOHME CORP. (MSD), WHITEHOUSE STATION, NEW JERSEY, UNITED STATES OF AMERICA

J. Dennie
D.E. Gutstein
P.-J. de Kam
R.B. Langdon
M.D. Troyer