Effects of SanOrg123781A, a synthetic heparin mimetic, in a mouse model of electrically induced carotid artery injury: synergism with the antiplatelet agent clopidogrel.

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Running title
Antithrombotic synergism of SanOrg123781A with clopidogrel

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Abbreviations

AT: anti-thrombin
PF4: platelet factor 4
HIT: heparin-induced thrombocytopenia
LMWH: low molecular weight heparin
TTO: Time to occlusion

Section
Cardiovascular
Abstract

SanOrg123781A is a synthetic hexadecasaccharide displaying antithrombin-dependent inhibition of factor Xa and thrombin and potent antithrombotic effects. The antithrombotic activity of SanOrg123781A has been studied in a new mouse model of arterial thrombosis, where thrombus formation was induced by application of an electric current to the advential surface of a carotid artery. In this model, antiplatelet agents such as the ADP-receptor antagonist clopidogrel (30 mg/kg, p.o. 2h before stimulation) and the GpIIb/IIIa antagonist SR121566A (0.3 mg/kg, i.v. 5 min before stimulation) strongly prolonged the time to occlusion (TTO, 761 and 473 % increases, respectively), whereas aspirin was devoid of antithrombotic activity. Standard heparin (2 mg/kg, i.v.), the low molecular weight heparin (LMWH) enoxaparin (20 mg/kg, i.v.), as well as the synthetic, antithrombin-dependent inhibitor of factor Xa fondaparinux (10 mg/kg, i.v.) were also active in this model (742, 707 and 602 % TTO increase, respectively). Interestingly, SanOrg123781A was active at much lower doses than the other oligosaccharides (554 % increase in TTO at 0.3 mg/kg, i.v. 5 min before stimulation). Low doses of SanOrg123781A administered in combination with low doses of clopidogrel led to a marked increase in TTO which was statistically more important than the additive effects of the two compounds given alone. These results indicate that SanOrg123781A exerts a potent antithrombotic activity in a mouse model of arterial thrombosis when compared to reference compounds and show that the combination of SanOrg123781A with clopidogrel leads to a marked synergistic antithrombotic effect.
Platelets and thrombin play crucial roles in the pathogenesis of arterial thrombotic diseases. Thus, antiplatelet and anticoagulant agents represent the cornerstones of the management for these events (Cairns et al., 2001). However, the effectiveness of traditional standard therapies (i.e. aspirin and heparin), is today not considered as optimal. This is due in part to the multiple and interdependent pathways involved in the process of thrombus formation. Thus, in addition to aspirin, new antiplatelet agents have been designed to provide greater protection. This includes the inhibitors of GpIIb/IIIa complex and the ADP-receptor antagonist clopidogrel, the latter of which has recently demonstrated an overall benefit in patients, treated with aspirin, presenting an acute coronary syndrome (Mehta and Yusuf, 2000). Heparin is known to exhibit potent antithrombotic effects chiefly through potentiation of antithrombin (AT), the major physiological inhibitor of several blood coagulation serine proteases, notably thrombin and factor Xa. Commercially available heparins are heterogeneous preparations of glycosaminoglycans which, in addition to AT, show non-specific affinity for endothelial cells, platelet factor 4 (PF4), and several plasma proteins, resulting in complex pharmacokinetics and adverse side effects such as heparin-induced thrombocytopenia (HIT). Although low molecular weight heparins (LMWH) demonstrate somewhat reduced non-specific interactions, they are still mixtures and retain some of the disadvantages of unfractionated heparin, albeit much attenuated (Warkentin at al., 1998, Warkentin, 1999, Eikelboom et al. 2000, Hirsh et al., 2001). The synthetic pentasaccharide fondaparinux (Arixtra®), which is structurally based on the minimal active sequence of heparin that binds AT, selectively inhibits factor Xa, lacks non-specific interactions and has been shown to be a potent antithrombotic drug in animal models as well as in clinical trials (Herbert et al., 1996, Herbert et al, 1998, Rembrandt study, 2000, Bauer et al., 2002). However, to obtain potent
inhibitors of both factor Xa and thrombin, longer oligosaccharides are needed. SanOrg123781A is the first example of a totally synthetic hexadecasaccharide exhibiting a mixed profile (AT-mediated inhibition of both factor Xa and thrombin activities) thus retaining the full antithrombotic properties of unfractionated heparin. In addition, SanOrg123781A, unlike heparin, does not interact with PF4 and does not activate human platelets in the presence of plasma from HIT patients (Herbert et al., 2001). SanOrg123781A has been the subject of several preclinical reports demonstrating that it exhibits prolonged anti-Xa and anti-thrombin activities, a high subcutaneous bioavailability as well as potent venous and arterial antithrombotic effects in rats and pigs (Herbert et al., 2001, Bal dit Sollier et al., 2001, Herault et al., 2002). Like heparin or LMWH, SanOrg123187A is likely to be administered together with antiplatelet agents in acute coronary syndromes and the effects of such a combination therapy have not yet been studied.

In the present work, we describe the characterisation of a new arterial thrombosis model in the mouse where thrombus formation is induced by application of an electric current to the advential surface of carotid artery. We have used this model to compare the activity of SanOrg12381 to other polysaccharides (standard heparin, LMWH and the synthetic pentasaccharide fondaparinux) and to antiplatelet agents such as clopidogrel or the GPIIb/IIIa inhibitor SR121566 (Badorc et al., 1997). In addition, the development of a simple and reproducible arterial thrombosis model in the mouse enabled us to perform a large association study using 16 different dose combinations of SanOrg123781A and clopidogrel in order to evaluate the potential synergistic effect of combined antithrombotic treatment.
Methods

Thrombosis induction

Male Balb/C mice (26-30g, Charles River, France) were anaesthetized (Sodium pentobarbitone 40 mg/kg + ketamine 40 mg/kg followed by 40 mg/kg/h, i.p), artificially ventilated (Hugo Sachs Apparatus Minivent type 845 respirator) and placed on a heated jacket to control body temperature (Harvard homeothermic blanket control unit). A femoral vein was cannulated for i.v. injections. A segment (approximately 0.5 cm long) of the right carotid artery was exposed and fitted distally with an appropriately sized Doppler flow probe (Soft Doppler Flow Cuff P/N E20-08 Triton Technology, San Diego, USA). A J-shaped bipolar stainless steel miniature electrode (Harvard Apparatus) was placed around the vessel, proximal to the probe and thrombosis was induced by applying a constant electrical current (1 mA) to the external arterial surface using a DC stimulator (Sanofi-Synthélabo, Chilly-Mazarin, France) for 90 s. This stimulation protocol was the minimum necessary to give reproducible artery occlusion. Blood velocity was measured and recorded on a chart recorder (Graphtec, Antony, France) for 60 minutes post-stimulation. Artery occlusion was defined as zero signal from the Doppler probe. When the flow declined to zero, the time to occlusion (TTO) was noted, as was the number of animals still presenting a flow signal 60 min post lesion. The Animal Care and Use Committee of Sanofi-Synthélabo Recherche has approved this protocol. All experiments are in accordance with the principles laid down in the European Convention for the Protection of Vertebrate Animals used for Experimental and other Scientific Purposes and its appendix.
Scanning electron microscopy

Scanning electron microscopy was performed on carotid artery samples from certain sham (carotid artery exposed to the electrode without stimulation) and electrically-stimulated (after 6 min stimulation) mice in order to characterise the structure of the arterial thrombus. In these animals, an intracardiac infusion was performed at a constant pressure (90 cm H₂O) using 0.9% saline during 5 min followed by 4% paraformaldehyde (5 min) before collecting artery segments. After longitudinal section, carotid samples were then fixed with 25% glutaraldehyde in cacodylate buffer (0.4 M, pH 7.2), before being rinsed with the same medium. Subsequent postfixation was in 2% osmium tetroxide in cacodylate buffer for 1 h at room temperature before dehydration in graded ethanol series and Freon followed by critical point drying (CPD020 Balzers). Samples were then coated with a 15 nm layer of gold before examination in a Philips 525 M scanning electron microscope.

Drugs

Sources of drugs used were as follows; ketamine (Virbrac, France), sodium pentobarbitone, aspirin lysine salt (Aspegic®), SR121566A, clopidogrel, fondaparinux (Arixtra®) and SanOrg123781A (Sanofi-Synthélabo, Toulouse/Chilly-Mazarin, France), enoxaparin (Aventis, France), heparin calcium salt (Sigma, Saint Louis, USA). In thrombosis experiments, drugs were administered in 0.9% saline for intravenous studies (0.1 ml/30 g, 5 min before stimulation) and in water for oral studies (0.1 ml/30 g, 120 min before stimulation). The development of SanOrg123781A and fondaparinux (Arixtra®) are being pursued within a partnership agreement between Sanofi-Synthélabo (Gentilly, France) and Organon (Oss, The Netherlands). Doses refer to the free bases.
Statistical analysis

For each treatment group, the mean TTO ± S.E.M. was determined, and tests for statistical significance between the treatment and control groups were performed by one way analysis of variance followed by the Logrank test using SAS software®. The percentage increase in TTO was determined for each treatment group. If the vessels were still patent at the end of the observation period, a value of 60 minutes was ascribed for the sake of statistical analysis. Groups were considered significantly different if \( p < 0.05 \). Synergy between clopidogrel and SanOrg123781A was assessed using a multivariate linear logistic model for two agents which estimates an interaction parameter (Greco et al., 1995) representative of the presence or absence of synergy or additivity. Synergy was considered significant if this parameter, including the confidence interval, was positive.
Results

Thrombotic occlusion induced by electrical injury of the mouse carotid artery

Application of an electric current (1 mA for 90s) to the adventitial surface of the right carotid artery quickly and reproducibly led to the formation of a stable occlusive thrombus denoted by zero flow signal (7.5 ± 0.6 and 7.0 ± 2 min in the groups pre-treated with i.v. saline, n=37, or p.o water, n=6, respectively). This thrombotic occlusion was stable for at least 60 min. Scanning electron microscopy of a longitudinal section of the sham artery revealed a normal endothelium with some scattered erythrocytes. The electrically-injured carotid artery exhibited an occlusive thrombus which was composed of dense platelet aggregate, protein deposits and significant clumps of erythrocytes. The distal (non-occlusive) part of the thrombus contained a fibrin network trapping blood cells, principally erythrocytes (Fig. 1).

Effects of anti-platelet agents and oligosaccharides on thrombus formation

Since platelets play a key role in arterial thrombosis, the effect of anti-platelet agents was assessed in this model. The effects of aspirin (0.1-100 mg/kg, i.v.), clopidogrel (3-30 mg/kg, p.o.) and SR121566A (0.1-1 mg/kg, i.v.) on TTO after electrical stimulation are shown in Figure 2. SR121566A and clopidogrel led to a dose-dependent increases in TTO, a statistically significant increase being observed at the doses of 0.3 mg/kg (473%) and 30 mg/kg (771%), respectively. It is noteworthy that aspirin did not modify TTO whatever the dose studied. The effect of oligosaccharides was also assessed. When SanOrg123781A and heparin were used, statistically significant increases in TTO were observed at the doses of 0.1 and 2 mg/kg (0.02 and 0.13 μmol/kg), respectively (Fig. 3 and 4). High doses of fondaparinux and
enoxaparin also led to significant increases in TTO (602 and 707 %) at 10 and 20 mg/kg (5.78 and 4.4 µmol/kg) (Fig. 4).

Effects of SanOrg123781A, and clopidogrel alone or in combination

In a second set of experiments, in order to study the potential interest of a combined treatment, several low doses of SanOrg123781A and clopidogrel were used alone or together. In total, 16 different dose combinations were tested. As shown in Fig. 5a, low doses of SanOrg123781A were without effect on TTO when used alone, but the addition of doses of clopidogrel, which were also inactive per se, resulted in a marked prolongation of TTO. Thus, 80% of vessels were still patent 1 h after electrical injury after the administration of SanOrg123781A (0.01 mg/kg) in combination with clopidogrel (10 mg/kg) (Fig. 5b). Simultaneous statistical analysis of all the data points (by fitting a multivariate linear logistic model to the data, Greco et al., 1995) showed that the compounds acted in synergy (interaction parameter $\beta_{12}: 5.0191$, confidence limits 1.5667; 8.4715).
Discussion

In arterial thrombosis, the inter-relationship which exists between endothelial injury, platelet aggregation and activation of the coagulation system, plays a major role in thrombus formation and has provided the impetus for the development of standardized thrombosis models using endothelial injury. Arterial thrombosis induced by perivascular electric stimulation (Hladovec, 1971) has been shown to be a suitable and relevant model for the discovery and the selection of antithrombotic drugs in several animal species. To our knowledge, this model has not yet been described in the mouse. The mouse has recently become an attractive species for pharmacological studies in the thrombosis area for two reasons. Firstly, the increasing availability of transgenic mice models of coagulation or platelet function disorders produced by gene knock-out or upregulation. Secondly, and this was particularly relevant to the present work, the small size of the mouse renders more feasible large scale multi-dose combination studies of the type we have performed using SanOrg23781A and clopidogrel which involved 16 experimental groups. Performing a similar study in a large animal species would necessitate evaluation of factors such as, animal availability, cost and quantity of compounds required. This latter point is a significant problematic in the case of the synthetic oligosaccharides such as SanOrg123781A because of the complexity of their syntheses.

In the present study, we show that application of an electric current around the external wall of the carotid artery results in arterial occlusion which occurs in a highly reproducible fashion approximately 7-8 minutes after the termination of the electrical stimulation, concomitantly with the formation of a mixed-type thrombus, as
demonstrated by electron microscopy. It is noteworthy that thrombus formation was inhibited by the antiplatelet drugs SR121566A and clopidogrel at doses which are in a similar range as those which have been shown to exert arterial antithrombotic effects in other species (Bernat et al., 1993, 1999; Herbert et al., 1998). We are not aware of other studies of the antithrombotic effect of clopidogrel in the mouse. However, our data are consistent with the demonstration that clopidogrel (25 mg/kg p.o.) markedly inhibits ADP-induced platelet aggregation in this species (Foster et al., 2001). In contrast, aspirin was devoid of antithrombotic activity over a wide range of doses (1 – 100 mg/kg i.v.), suggesting that the mouse is resistant to the antithrombotic effects of aspirin. A similar lack of activity of aspirin has been described in several rat models of arterial thrombosis (Schumacher et al., 1993; Lockyer and Kambayashi, 1999). Given that André et al. (2003) report that aspirin (10 mg/kg i.v.) is sufficient to completely block arachidonic acid-induced aggregation of mouse platelets, we believe that the dosing regimen that we have adopted for aspirin covers the pharmacological range. In our study, enoxaparin was poorly active. This has already been reported in a ferric chloride-induced arterial thrombosis model in the rat (Toomey et al., 2000). In contrast, SanOrg123781A displayed strong antithrombotic effects which were greater than those observed for heparin or fondaparinux, in accordance with reports from other thrombosis models (Herbert et al., 2001; Bal dit Sollier et al., 2001).

A key objective of the present study was to evaluate the arterial antithrombotic effects of the oligosaccharide SanOrg123781A alone and in combination with the antiplatelet agent clopidogrel. This combination is of particular relevance because the current state of the art in the treatment of arterial thrombotic disorders,
particularly acute coronary syndromes, involves the combination of antiplatelet and anticoagulant agents (Cairns et al., 2001). Among the antiplatelet drugs, aspirin is used extensively in these pathologies, but is ineffective in blocking multiple pathways of platelet activation, emphasizing the need for other antiplatelet agents of superior efficacy (Cairns et al., 2001). This led to the development of the ADP-receptor antagonist clopidogrel, which has been proven effective in patients presenting atherosclerotic vascular disease or an acute coronary syndrome (CAPRIE study 1996; Mehta and Yusuf, 2000). The early antithrombotic management of unstable angina and non-Q-wave acute myocardial infarction is routinely completed by heparin or LMWH. The anticoagulant activity of these compounds is largely due to their ability to produce a conformation change in AT, followed by the inactivation of serine protease clotting factors (mainly factor Xa and thrombin). However, heparin and, to a lesser extent, LWMH, in addition to binding to AT, interact with other biological molecules unrelated to the coagulation process resulting in variable bioavailability and side effects (Cornelli et al., 1999; Warkentin et al., 1998; Warkentin, 1999; Eikelboom et al., 2000; Thomas, 1997). SanOrg123781A, produced by total chemical synthesis, shares the dual anticoagulant activity of heparin, acting on factor Xa and thrombin through AT binding, without non-specific effects (no interaction with PF4) and demonstrates excellent pharmacokinetic characteristics (Herbert et al., 2001). It extends the therapeutic characteristics arsenal of synthetic antithrombotic oligosaccharides, of which the pentasacharide fondaparinux (Arixtra®) is the spearhead (Bauer et al., 2002). In view of its potential advantages over heparin, SanOrg123781A would be expected to be of interest in the treatment of arterial thrombosis.
The potent antithrombotic effects of clopidogrel and SanOrg123781A, alone, observed in our mouse model, justified a study of combined administration with low doses of both compounds. Our data demonstrated that the co-administration of SanOrg123781A and clopidogrel was associated with greater antithrombotic effects than would be expected from the simple addition of the respective antithrombotic activities of these compounds. In fact, whereas an additive effect of an antiplatelet drug like clopidogrel and an anticoagulant compound like SanOrg1237812A would not be totally unexpected, the synergy observed between these compounds came as a surprise. Part of the explanation may be related to the effect of clopidogrel on thrombin generation in platelet rich plasma (Herault et al., 1999), which emphasises the inter-relationship that exists between platelet activation and coagulation pathways. Even limited platelet inhibition by low doses of clopidogrel may decrease coagulation activation sufficiently to favour AT-dependent inhibition. However, complete elucidation of the mechanism of synergy which occurs between clopidogrel and SanOrg123781A will require further studies.

In conclusion, electrical injury-induced arterial thrombosis in the mouse carotid artery provides a robust and reproducible thrombosis model which shares the characteristics of the "classical" models of arterial thrombosis: it is sensitive to anti-aggregating agents like clopidogrel or GpIIb/IIIa inhibitors as well as to oligosaccharidic antithrombotic drugs. In this model, SanOrg123781A demonstrates a potent antithrombotic activity alone and a strong synergistic effect when administered in combination with the antiplatelet agent clopidogrel. These data suggest that combined treatment with SanOrg123781A and clopidogrel may be particularly interesting in the context of arterial thrombotic diseases and notably in
acute coronary syndromes, where SanOrg123187A is likely to be administered together with antiplatelet agents.

Acknowledgments

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References


Footnotes

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Figure legends

**Figure 1:** Scanning electron photomicrographs of the surface of the carotid artery after sham treatment (a) and after electrically-induced injury (b and c). In (a), the intact endothelium is visible, associated in places with scattered red blood cells. Fig. 1b shows proximal part of an occlusive thrombus consisting of very dense platelet aggregates covered by proteinaceous material and clumps of erythrocytes. Fig. 1c shows the distal section (non-occlusive) of the same thrombus with erythrocytes and a few platelets trapped in a fibrin network.

**Figure 2:** Effect of antiplatelet agents: clopidogrel (3 - 30 mg/kg p.o., black histograms) SR121566A (0.1 – 1.0 mg/kg i.v., hatched histograms) and aspirin (0.1-100 mg/kg, i.v., white histograms) on time to occlusive thrombus formation (TTO) in the mouse carotid artery model. Compounds were given either 5 min (SR121566A, aspirin) or 120 min (clopidogrel) before arterial lesion. The numbers within each histogram (e.g. 0/7) refer to the number of animals in each group showing a patent carotid artery 60 min after arterial lesion. **p<0.01 versus vehicle group.

**Figure 3:** Effect of SanOrg123781A (0.03 – 1.0 mg/kg i.v.) on time to occlusive thrombus formation (TTO) in the mouse carotid artery model. SanOrg123781A was given 5 min before arterial lesion. The numbers within each histogram (e.g. 0/7) refer to the number of animals in each group showing a patent carotid artery 60 min after arterial lesion. *p<0.05, **p<0.01 versus vehicle group.
Figure 4: Effects of oligosaccharides: fondaparinux (1 to 10 mg/kg, i.v., black histograms), heparin (0.1 to 3.0 mg/kg i.v., hatched histograms), and enoxaparin (1 to 20 mg/kg, i.v. white histograms) on time to occlusive thrombus formation (TTO) in the mouse carotid artery model. Compounds were given 5 min before arterial lesion. The numbers within each histogram (e.g. 0/6) refer to the number of animals in each group showing a patent carotid artery 60 min after arterial lesion. ** p<0.01 versus vehicle group.

Figure 5: Effect of SanOrg123781A (0.01, 0.03, 0.1 mg/kg, i.v.) alone or in combination with clopidogrel (CL, 1, 3, 10 mg/kg p.o.) on time to occlusive thrombus formation (TTO) in the mouse carotid artery model (a) and % vessel patency (b). The clopidogrel alone groups correspond to the symbols at point “0” on the SanOrg123781A axis. Each symbol shown denotes a single experimental group (16 in total, n=10 per group). ** p<0.01 versus vehicle group.
Figure 2

[Bar chart showing TTO (min) vs. dose (mg/kg, p.o. and mg/kg, i.v.) for different conditions (H₂O, saline, and various doses).]
Figure 3

![Graph showing the effect of SanOrg123781A on TTO (min) for different doses and saline control.](image-url)

SanOrg123781A (mg/kg, i.v.)

- Saline: 0/7
- 0.03 mg/kg: 0/7
- 0.1 mg/kg: 2/7
- 0.3 mg/kg: 5/7
- 1 mg/kg: 6/7

*P < 0.05, **P < 0.01
Figure 4

The figure shows a bar graph comparing the TTO (min) at different mg/kg, i.v. levels of saline and other treatments. The graph includes error bars indicating variability. The treatments are labeled with concentrations in mg/kg, i.v., and the Y-axis represents TTO in minutes. The graph includes statistical significance markings (** and ***).
Figure 5

- ● SanOrg123781A alone
- △ + CL 1 mg/kg
- ▼ + CL 3 mg/kg
- □ + CL 10 mg/kg

(a) TTQ (min)

(b) % vessel patency at 60 min

SanOrg123781A mg/kg, i.v.
Figure 2
Figure 3

![Graph showing TTO (min) vs SanOrg123781A (mg/kg, i.v.)]

- Saline: 0/7
- 0.03 mg/kg: 0/7
- 0.1 mg/kg: 2/7
- 0.3 mg/kg: 5/7
- 1 mg/kg: 5/7

**Significant differences:**
- *: p < 0.05
- **: p < 0.01
Figure 4

[Graph showing TTO (min) vs. mg/kg, i.v. for different concentrations of saline (0.6, 0.5, 0.1, and 0.01) and different dosages (1, 3, 10, and 20 mg/kg, i.v.).]
Figure 5

- SanOrg123781A alone
- △ + CL 1 mg/kg
- ▽ + CL 3 mg/kg
- ■ + CL 10 mg/kg

**TTO (min)**

- **a**
- **b**

**% vessel patency at 60 min**

SanOrg123781A mg/kg, i.v.