Inhibition of factor IXa by the pegnivacogin system during cardiopulmonary bypass: a potential substitute for heparin.

A study in baboons

Alain Bel, Wasseem Borika, Simon Davidsonb, Jean-Marie Heliesc, Lev Stimmerd, Stephen Fremese,
Steven Zelenkofske, Christopher Rusconi, John Alexanderg, David Alexanderh, Philippe Menaschéa and John Pepperh,*

a Assistance Publique-Hôpitaux de Paris, Hôpital Européen Georges Pompidou Department of Cardiovascular Surgery, Paris, France
b Department of Haematology, Royal Brompton Hospital, London, UK
c Molecular Imaging Research Center, Commissariat à l’Energie Atomique, Fontenay-aux-Roses, France
d CEA-INSERM U986, Commissariat à l’Energie Atomique, Fontenay-aux-Roses, France
e Department of Cardiovascular Surgery, Sunnybrook Health Sciences Centre, Toronto, ON, Canada
f Regado Biosciences, Basking Ridge, NJ, USA
g Cardiovascular Thrombosis, Duke Clinical Research Institute, Durham, NC, USA
h Department of Cardiothoracic Surgery, Royal Brompton Hospital, London, UK
i Université Paris Descartes, Sorbonne Paris Cité; INSERM U 970, Paris, France

* Corresponding author. NIHR Biological Research Unit, Royal Brompton Hospital, London SW3 6NP, UK. Tel: 44-207-3518530; fax: 44-207-3518530; e-mail: j.pepper@rbht.nhs.uk (J. Pepper).

Received 20 January 2015; received in revised form 19 March 2015; accepted 24 March 2015

Abstract

OBJECTIVES: Heparin and protamine are standard for anticoagulation and reversal for cardiopulmonary bypass (CPB). The REGADO biosciences protocol 1 (REG1) anticoagulant system, consisting of the Factor IXa (FIXa)-inhibitor pegnivacogin and its reversal agent (anivamersen), has been studied in patients undergoing coronary catheterization and in CPB in sheep and pigs. Prior to first human use in CPB, we wanted to test the safety and efficacy of REG1 in a primate model.

METHODS: Fourteen baboons undergoing 2 h of CPB followed by 1 h of reperfusion were studied. Three received heparin/protamine and 11 received 1 of 2 doses of pegnivacogin followed by anivamersen. Thrombin-generating capacity was tested in additional in vitro experiments.

RESULTS: Targeted drug levels and near-complete FIXa inhibition were achieved. Bypass was run uneventfully in all animals without any clotting in the circuit and bleeding was minimal in the two groups. However, in contrast to heparin-treated baboons, those receiving pegnivacogin/anivamersen displayed thrombi in the bypass cannulae upon cannulation and kidney cortical infarcts. Inter-species comparisons revealed that in the presence of high levels of FIXa inhibition, tissue factor-mediated thrombin generation in baboons was much higher than that in other species.

CONCLUSIONS: These data highlight the limitations of the baboon model for assessing factor-specific coagulation inhibitors during CPB. The justification for Phase 1 human studies using REG1 for CPB is unclear.

Keywords: Factor IX • Cardiopulmonary bypass • Anticoagulation • Thrombin generation • Peginvagocin/Anivamersen

INTRODUCTION

Intravenous unfractionated heparin followed by protamine is routinely used for anticoagulation and reversal, respectively, in patients undergoing cardiac surgery both with and without cardiopulmonary bypass (CPB). Unfortunately, heparin does not fully inhibit thrombin generation and therefore may contribute to haemostatic activation, particularly during CPB, leading to subsequent adverse ischaemic and bleeding events, defined as the need for transfusion or reoperation and associated with worse outcomes and higher costs of treatment [1, 2]. In addition, protamine reversal itself may be independently associated with adverse clinical events including anaphylactic reactions, severe systemic hypotension, bronchospasm, pulmonary vasoconstriction or even death [3].

For these reasons, and despite the continued development of novel antithrombics, a need exists for safer anticoagulants in the setting of cardiac surgery and prolonged cardiac assistance, as
achieved by ventricular assist devices and extracorporeal membrane oxygenation. The ideal intravenous anticoagulant for cardiac surgery would be immediately effective, reliably monitored, easily titrated to a level that would prevent thrombosis and, be immediately and predictably reversible.

The REGADO biosciences protocol 1 (REG1) anticoagulation system is a novel, first-in-class anticoagulation system consisting of a drug component (pegnivacogin) and the active control agent specific to pegnivacogin (anivamersen). Peggivacogin is an oligonucleotide aptamer that elicits an anticoagulant effect by blocking the Factor VIIIa/Factor IXa (FIXa) catalyzed conversion of Factor X to Factor Xa. Anivamersen is an oligonucleotide complementary to a portion of pegnivacogin that can effectively bind to pegnivacogin and thereby neutralize its anti-FIXa activity.

Following Phase 2 studies performed in the setting of cardiac catheterization, with or without percutaneous coronary intervention (PCI), and which, overall, have confirmed the safety and suggested the efficacy of the REG1 system [4, 5], it seemed wise to test the drug and its reversal agent during clinical open heart surgery (OHS). Two prior studies in CPB were performed in sheep and pigs successfully. However, before considering implementation of REG1 during OHS, an additional animal study was deemed necessary to gain additional non-clinical experience with the drug prior to its use in humans and to provide additional safety data.

MATERIALS AND METHODS

All animal experiments were conducted in a state-of-the-art preclinical surgical facility with immediate access to a purpose-bred baboon colony. The animals were individually housed in separate cages conforming to the standards set forth in The Guide for the Care and Use of Laboratory Animals. Baboons were fed a certified constant-nutrient diet with tap water being provided ad libitum. Environmental controls were set to maintain temperatures of 16–27°C and lighting controls were set to maintain a 12:12 h light/dark cycle. Temperature and humidity were monitored continuously by a centralized system.

Surgical procedures

Fourteen (9–12-year-old) female Papio Anubis baboons weighing 11–14 kg, were anaesthetized with intramuscular (IM) injections of ketamine and xylazine (10 + 0.5 mg/kg). Prior to anaesthetic induction, the animals received glycopyrrolate (0.01 mg/kg, IM) to prevent hypersalivation and bradycardia. After local anaesthesia of the larynx, the animals were intubated and maintained with intravenous propofol (0.1–0.2 mg/kg min) and sufentanil (0.1–0.3 µg/kg/h) adjusted to effect, for analgesia. Under general anaesthesia, both femoral vessels were cannulated for insertion of intravenous and intra-arterial lines for collection of baseline samples and fluid replacement, as necessary. Through a mid-line sternotomy, CPB was then established between a two-stage 28F venous straight cannula (Edwards), and a 16F angled cannula (Terumo) placed into the ascending aorta. Each CPB circuit was assembled, using the Terumo paediatric pack (Terumo CxTP 70 83) tubing incorporating an oxygenator (RX15), an arterial filter (32 µ), a roller pump and an open venous reservoir. Priming consisted of a 600-ml volume of saline and macromolecules [Voluven®: 6% hydroxyethyl starch 130/0.4 in 0.9% sodium chloride injection. Fresenius, Australia]. Animals were anticoagulated with heparin or pegnivacogin directly into the right atrium prior to establishment of CPB, and anticoagulant was added to the pump prime as well (detailed below). Normothermic CPB was run at a flow rate of 100 ml/kg/min for 2 h with the heart kept beating (no cardioplegia). A suction cannula was available for removal of blood from the pericardial cavity. The animals were weaned from CPB, the cannulae were removed and the reversal agent (detailed below) was administered. The animals were observed for a further 1 h before receiving a lethal injection in accordance with the institutional veterinary practice (overdose of intravenous sodium pentobarbital).

Periprocedural samples

Arterial haematology blood count measurements included standard parameters. Standard serum chemistry measurements were collected pretreatment as part of the health assessment of the animals upon arrival at the testing facility. For pharmacokinetic (PK) studies, blood samples were collected in K3EDTA tubes, placed on wet ice immediately and centrifuged under refrigeration (2–8°C) for 10 min at 3500 rpm within 30 min of collection and stored at −70°C until analysis. Peggivacogin plasma concentrations were measured, using a dual hybridization Enzyme-linked immunosorbent assay (ELISA) assay as previously described (PPD Richmond, VA, USA) [6]. This method was qualified for use in baboon plasma for specificity, working calibration range, linearity, accuracy and intrabatch precision. The linear range for the assay was 10.0–200 ng/ml. Method performance during data collection, as determined by analysis of quality control (QC) samples included on each plate, was suitable. PK samples from heparin-treated animals were collected and processed identically to those obtained from REG1-treated animals, but were not tested.

Experimental groups

Three animals received unfractionated heparin (Héparine Choay®, Sanofi Aventis, France) given at the dose of 300 IU/kg in addition to 5000 IU heparin (1 ml) in the pump prime and followed by reversal with protamine in a 1:1 ratio. Eleven animals received pegnivacogin and reversal with anivamersen. Among them, six received a dose of 1.5 mg/kg and five received a higher dose of 2.5 mg/kg. In addition, the pump prime was dosed with pegnivacogin (40 and 67 µg/ml final concentration for animals treated with 1.5 and 2.5 mg/kg pegnivacogin, respectively) to match the anticipated blood levels of the drug. Anivamersen was administered at a weight:weight dose ratio to pegnivacogin of 1:1 and 0.5:1 ratio in 4 and 2 1.5 mg/kg-treated baboons, respectively (including pegnivacogin administered to the CPB pump prime). In the group of five baboons receiving 2.5 mg/kg pegnivacogin, the anivamersen/pegnivacogin ratio was 1:1 in 2 and 0.5:1 in 3 animals.

Prior studies have demonstrated that pegnivacogin plasma concentrations ≥15 µg/ml, achieved by a dose of ≥0.7 mg/kg, provide >99% inhibition of FIXa activity [5, 6]. Although near-complete inhibition of FIXa occurs at pegnivacogin plasma concentrations >15 µg/ml, pegnivacogin concentrations >20 µg/ml were targeted for the acute coronary syndrome and OHS studies to achieve levels that are on the upper plateau of FIXa inhibition. Therefore, a 1.5 mg/kg dose of pegnivacogin, anticipated to result in a maximum plasma concentration of ~40 µg/ml [6], was chosen to compensate for volume expansion during CPB in order to...
maintain plasma concentrations >20 µg/ml and to be consistent with planned pegnivacogin dosing in OHS clinical studies. After observation of thrombi in the cannula during the first set of the present experiments, the 2.5 mg/kg pegnivacogin dose was implemented as the dose–response relationship to pegnivacogin had not been previously established in baboons.

Selection of anivamersen doses was based on the observed anivamersen dose–response in the REG1 Phase 1 and 2 Programs, where it is anticipated that reversals of 75% (0.5:1 ratio of anivamersen: pegnivacogin) will result in near normalization of coagulation and 100% reversal (1:1 dose ratio) will result in full normalization of coagulation.

Assessment of outcomes

Thrombus assessment. The venous reservoir and oxygenator were observed for the presence of thrombus at ~15-min intervals during CPB. The chest cavity was also evaluated periodically during CPB for the presence of clots. Upon completion of the CPB procedure, the oxygenator was flushed with saline and observed again for the presence of thrombus.

Bleeding assessment. A pericardial drain was placed for collection of intraoperative blood losses and fluids retrieved from the chest cavity were not recirculated through the bypass circuit (except for one REG1-treated and one heparin-treated animal). In addition, a retrosternal drain was used to measure blood losses over the post-bypass 1-h observation period.

Haemostasis assessment. Time to haemostasis following administration of reversal agent was assessed by measuring the time from administration of reversal agent to time of skin closure.

Pathological and histological assessment. Immediately after euthanasia, all animals underwent necropsy by specially trained veterinary pathologists. All organs were examined macroscopically to identify any lesions or abnormalities. Representative slides were then prepared for microscopic examination with haematoxylin–eosin staining from the brain, heart, kidneys, liver, lungs, adrenal gland, spleen and pancreas from each animal. Kidney and lung sections as well as sections from tissue in which thrombus was observed upon macroscopic and microscopic examination were further evaluated by staining for platelets (anti-CD61; Dako, France) and fibrinogen (antifibrin polyclonal serum; Dako, France) followed by immunohistochemical evaluation.

Observed microscopic lesions were graded according to a severity score attributed according to the size and number of observed lesions based on the following scale: ‘0’: within physiological limits; ‘1’: minimal; ‘2’: mild; ‘3’: moderate and ‘4’: severe. Individual scores for each animal were provided by organ class. To enable group comparison, a mean severity score was then calculated as the arithmetic mean of scores for each parameter by organ class for haemorrhage severity and for thrombus severity.

In vitro coagulation samples

An additional set of samples were collected from baboon, sheep, pig and human blood for in vitro comparison of pegnivacogin anticoagulant activity across species. In the inter-species comparison of thrombin-generating capacity, human, pig and sheep blood displayed close patterns with regard to blockade of thrombin generation in response to given doses of pegnivacogin whereas, at those same doses, baboons were unexpectedly found to be still able to generate substantial quantities of thrombin (Fig. 1A and B).

Samples were centrifuged at 1800–2000 g for 10 min to prepare platelet-poor plasma that was then stored as 0.5 ml aliquots at ~80°C and shipped frozen to the Department of Haematology at the Royal Brompton and Harefield National Health Service Foundation Trust in London for spiking measurements of thrombin generation. Thrombin generation was performed using a fluorogenic Thrombin Generation Assay, TGA (Technoclone Diagnostics, Vienna, Austria). Briefly, 40 µl of citrated plasma was dispensed into a 96-well ELISA plate that had been prewarmed to 37°C (NUNC F16 MaxiSorp black fluorescence plates, Pathway Diagnostics, Dorking, UK). Added to this was 2 or 5 µl of drug stock solution (21 mg/ml) plus 10 µl tissue factor at a final concentration of 4.75pM (Technoclone Diagnostics) followed by 50 µl of the fluorogenic substrate 1 mM Z-G-G-R-AMC (Technoclone Diagnostics). The plate was loaded into the fluorogenic plate reader.
TECAN infinite F200 pro (Labtech International, Uckfield, UK), and measurement made every 60 s for a total of 1 h. The TGA software was used to calculate individual thrombin generation curves. The assay was calibrated, and QC performed according to the manufacturer’s instructions.

RESULTS

Pharmacokinetics

Pegnivacogin plasma concentrations were measured predose, post-dose prior to initiation of CPB, during CPB and 15 min post-administration of anivamersen (Tables 1 and 2). As given in Table 2, the pegnivacogin plasma concentration achieved at both doses was modestly higher in the baboon than as anticipated from prior studies in humans: 49.9 vs 40 µg/ml and 89.7 vs 67 µg/ml for the 1.5 and 2.5 mg/kg doses, respectively (Sample 2). As a result, the plasma concentration initially decreased upon establishment of CPB (Sample 3) as the pegnivacogin concentration in the pump prime was lower than that achieved after IV administration to the baboons. Subsequently, the plasma concentrations remained relatively unchanged throughout the remaining time on CPB (Sample 5), as expected given the molecular weight of pegnivacogin.

Administration of anivamersen at a dose ratio of 1:1 or 0.5:1 markedly reduced the plasma concentration of pegnivacogin independent of the absolute pegnivacogin dose, with the 1:1 dose ratio performing as expected and the 0.5:1 dose ratio of anivamersen providing a greater level of reversal than anticipated from prior studies in humans [7].

Coagulation measures

Baseline coagulation characteristics of the baboons were measured (n = 14) preoperatively for activated clotting time (ACT), activated partial thromboplastin time (aPTT), prothrombin time (PT) and fibrinogen (Table 3). Additional samples for thromboelastography (TEG) analysis were collected from nine REG1-treated baboons. After dosing with pegnivacogin at either 1.5 or 2.5 mg/kg, the aPTT prolonged immediately by a factor of 3.2–3.7 from baseline. Upon initiation of CPB (Sample 3), aPTT increased further, likely related to haemodilution of the animals due to CPB, as evidenced by the large decrease in fibrinogen concentration.

Table 1: Pegnivacogin plasma concentration by the dose group

<table>
<thead>
<tr>
<th>Sample</th>
<th>1.5 mg/kg pegnivacogin (µg/ml)</th>
<th>2.5 mg/kg pegnivacogin (µg/ml)</th>
<th>1:1 (w:w) anivamersen:pegnivacogin (µg/ml)b</th>
<th>0.5:1 (w:w) anivamersen:pegnivacogin (µg/ml)b</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>BLQ</td>
<td>BLQ</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>49.9 ± 6.6</td>
<td>89.7 ± 6.0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>44.0 ± 3.8</td>
<td>68.2 ± 7.0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>37.7 ± 7.9</td>
<td>57.9 ± 7.8</td>
<td></td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>0.03 ± 0.03</td>
<td>0.3 ± 0.3</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

aSample 1 = predose; 2 = 10 min post-pegnivacogin dose, pre-CPB; 3 = 15 min on CPB; 5 = 2 h on CPB; 6 = 15 min postanivamersen dose.
BLQ = below the limit of quantitation; <0.02 µg/ml
bFor post-reversal samples with a reported measure of BLQ, the BLQ value was used to calculate means and SD for the respective anivamersen dose groups.
Five of 6 animals in the 1:1 and two of five animals in the 0.5:1 dose group had a BLQ value for Sample 6.

Table 2: Coagulation parameters for REG1-treated animals by the dose group

<table>
<thead>
<tr>
<th>Sample</th>
<th>ACT (s)</th>
<th>ACT (ratio)</th>
<th>aPTT (s)</th>
<th>aPTT (ratio)</th>
<th>PT (s)</th>
<th>Fibrinogen (g/l)</th>
<th>% change in fibrinogen</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.5 mg/kg pegnivacoginb</td>
<td>110 ± 13</td>
<td>1 ± 0</td>
<td>41 ± 2</td>
<td>1 ± 0</td>
<td>16 ± 0.8</td>
<td>1.9 ± 0.4</td>
<td>NA</td>
</tr>
<tr>
<td>2</td>
<td>159 ± 32</td>
<td>1.4 ± 0.3</td>
<td>131 ± 54</td>
<td>3.2 ± 1.3</td>
<td>16 ± 1.0</td>
<td>1.7 ± 0.4</td>
<td>13 ± 9</td>
</tr>
<tr>
<td>3</td>
<td>217 ± 12</td>
<td>2.0 ± 0.2</td>
<td>160 ± 14</td>
<td>3.9 ± 0.2</td>
<td>19 ± 2</td>
<td>0.8 ± 0.2</td>
<td>59 ± 6</td>
</tr>
<tr>
<td>4</td>
<td>236 ± 25</td>
<td>2.2 ± 0.4</td>
<td>169 ± 30</td>
<td>4.1 ± 0.5</td>
<td>19 ± 0.5</td>
<td>0.8 ± 0.2</td>
<td>59 ± 7</td>
</tr>
<tr>
<td>5</td>
<td>243 ± 13</td>
<td>2.3 ± 0.3</td>
<td>176 ± 42</td>
<td>4.3 ± 0.8</td>
<td>20 ± 0.8</td>
<td>0.7 ± 0.2</td>
<td>64 ± 8</td>
</tr>
<tr>
<td>2.5 mg/kg pegnivacoginb</td>
<td>107 ± 8</td>
<td>1 ± 0</td>
<td>40 ± 5</td>
<td>1 ± 0</td>
<td>18 ± 1.3</td>
<td>1.7 ± 0.3</td>
<td>NA</td>
</tr>
<tr>
<td>2</td>
<td>178 ± 34</td>
<td>1.7 ± 0.4</td>
<td>144 ± 61</td>
<td>3.7 ± 1.7</td>
<td>17 ± 1.3</td>
<td>1.6 ± 0.3</td>
<td>7 ± 4</td>
</tr>
<tr>
<td>3</td>
<td>262 ± 24</td>
<td>2.5 ± 0.3</td>
<td>184 ± 43</td>
<td>4.6 ± 0.6</td>
<td>21 ± 1.1</td>
<td>0.6 ± 0.1</td>
<td>61 ± 6</td>
</tr>
<tr>
<td>4</td>
<td>254 ± 24</td>
<td>2.4 ± 0.1</td>
<td>161 ± 38</td>
<td>4.0 ± 0.7</td>
<td>20 ± 1.7</td>
<td>0.7 ± 0.1</td>
<td>59 ± 5</td>
</tr>
<tr>
<td>5</td>
<td>275 ± 40</td>
<td>2.6 ± 0.4</td>
<td>141 ± 36</td>
<td>3.5 ± 0.6</td>
<td>21 ± 1.1</td>
<td>0.6 ± 0.1</td>
<td>61 ± 6</td>
</tr>
<tr>
<td>1:1 (weight:weight) anivamersen:pegnivacogin</td>
<td>154 ± 21</td>
<td>1.4 ± 0.2</td>
<td>72 ± 19</td>
<td>1.9 ± 0.6</td>
<td>23 ± 2</td>
<td>0.7 ± 0.2</td>
<td>60 ± 10</td>
</tr>
<tr>
<td>0.5:1 (weight:weight) anivamersen:pegnivacogin</td>
<td>149 ± 14</td>
<td>1.4 ± 0.2</td>
<td>89 ± 19</td>
<td>2.1 ± 0.3</td>
<td>24 ± 0.9</td>
<td>0.7 ± 0.1</td>
<td>59 ± 6</td>
</tr>
</tbody>
</table>

aSample 1 = predose; 2 = 10 min post-anticoagulant dosing, pre-CPB; 3 = 15 min on CPB; 5 = 2 h on CPB; 6 = 15 min post-reversal agent dosing.
bData presented as means ± standard deviation with n = 4–6 per measurement.

ACT: activated clotting time; aPTT: activated partial thromboplastin time; PT: prothrombin time.
observed at this sampling time. The ACT followed a similar trend as the aPTT. The ACT increased by a ratio of 1.4–1.7-fold post-pegnivacogin administration, and increased further upon initiation of CPB. As expected for a direct FXa inhibitor, the PT was not impacted by pegnivacogin administration (Sample 2) but did increase slightly upon initiation of CPB, likely related to haemodilution. The fibrinogen was normal predose and showed no change.

Thromboelastography (TEG) parameters for 9 pegnivacogin-treated baboons

<table>
<thead>
<tr>
<th>Sample</th>
<th>r time (min)</th>
<th>k (min)</th>
<th>MA (mm)</th>
<th>% change</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>3.1 ± 1.27</td>
<td>1.0 ± 0.19</td>
<td>72.7 ± 5.3</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>13.0 ± 19.3</td>
<td>3.1 ± 1.7</td>
<td>62.8 ± 9.6</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>26.9 ± 8.3</td>
<td>5.6 ± 2.5</td>
<td>47.1 ± 6.5</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>29.0 ± 8.3</td>
<td>6.2 ± 2.7</td>
<td>44.8 ± 7.2</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>4.5 ± 1.3</td>
<td>1.6 ± 0.3</td>
<td>56.5 ± 5.3</td>
<td></td>
</tr>
</tbody>
</table>

aData presented as means ± standard deviation, n = 9.

Thromboelastography (TEG) parameters for 9 pegnivacogin-treated baboons

<table>
<thead>
<tr>
<th>Sample</th>
<th>ACT (s)</th>
<th>PT (s)</th>
<th>Fibrinogen (g/l)</th>
<th>% change in fibrinogen</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>108 ± 2</td>
<td>18 ± 1</td>
<td>2.7 ± 0.2</td>
<td>NA</td>
</tr>
<tr>
<td>2</td>
<td>529 ± 306</td>
<td>24 ± 2</td>
<td>1.9 ± 0.6</td>
<td>27 ± 27</td>
</tr>
<tr>
<td>3</td>
<td>458 ± 96</td>
<td>34 ± 4.7</td>
<td>0.9 ± 0.3</td>
<td>66 ± 13</td>
</tr>
<tr>
<td>4</td>
<td>374 ± 14</td>
<td>30 ± 3</td>
<td>0.8 ± 0.2</td>
<td>71 ± 10</td>
</tr>
<tr>
<td>5</td>
<td>387 ± 33</td>
<td>28 ± 1.5</td>
<td>0.8 ± 0.3</td>
<td>69 ± 13</td>
</tr>
<tr>
<td>6</td>
<td>141 ± 27</td>
<td>25 ± 2.5</td>
<td>0.7 ± 0.2</td>
<td>69 ± 8</td>
</tr>
</tbody>
</table>

aData presented as means ± standard deviation, n = 2–3 per measurement.

Table 5: Blood loss measures by the treatment and dose group

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Heparin/protamine</th>
<th>1.5 mg/kg pegnivacogin</th>
<th>2.5 mg/kg pegnivacogin</th>
<th>1:1 (w:w) anivamersen: pegnivacogin</th>
<th>0.5:1 (w:w) anivamersen: pegnivacogin</th>
</tr>
</thead>
<tbody>
<tr>
<td>Intraoperative blood loss (ml)</td>
<td>450 ± 210</td>
<td>97 ± 69</td>
<td>28 ± 48</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Postoperative blood loss (ml)*</td>
<td>15 ± 10</td>
<td></td>
<td></td>
<td>7 ± 11</td>
<td>9 ± 8</td>
</tr>
<tr>
<td>Time to chest closure (min)</td>
<td>16 ± 12</td>
<td></td>
<td></td>
<td>11 ± 9</td>
<td>8 ± 6</td>
</tr>
</tbody>
</table>

aData presented as means ± standard deviation with n = 2–3 per measurement for heparin-treated animals and 4–6 per measurement for REG1-treated animals.

Thrombotic assessments

CPB was uneventful in all animals independent of treatment, with all CPB runs going to planned completion of 2 h on CPB and with no clots observed in the circuit, oxygenator or reservoir, with one 10 min postadministration of pegnivacogin or heparin, noting however that limited published data indicate Clauss fibrinogen levels are lower in baboons than in humans. Fibrinogen levels decreased significantly, by ~60%, upon initiation of CPB and remained unchanged throughout CPB. This change is attributable to haemodilution, and similar changes were observed in blood counts [red blood cells (RBC), white blood cells (WBC), platelets; data not shown]. TEG showed normal or trend towards hypercoagulable results pre-REG1 for all animals (Table 4). Following administration of REG1, there was an increase in the r time and reduction in the maximum amplitude. The r time remained prolonged during the procedure and was still prolonged 8-fold after an hour on CPB.

Administration of heparin resulted in the anticipated increase in ACT values to generally >400 s as targeted in the protocol, with more modest changes observed in PT (Table 5). aPTT values for heparin-treated animals were all above the maximum reporting limit for the assay (data not shown).

Following the 2 h of bypass, anticoagulation was reversed and the above parameters were remeasured 15 min postadministration of the reversing agent (Sample 6). In REG1-treated animals, administration of anivamersen at a 0.5:1 or 1:1 dose ratio to pegnivacogin resulted in a significant decrease in ACT and aPTT, when compared with the last measure taken while the animals were on CPB, with no dose-related difference in reversal. Additionally, neither parameter returned fully to baseline despite the presence of only negligible levels of pegnivacogin in the plasma (Table 3).

The failure of anivamersen to restore ACT and aPTT to baseline levels is therefore most likely due to haemodilution of the animals, as suggested by the 60% decrease in fibrinogen levels and blood counts relative to baseline values. By comparison, TEG showed the r time fully corrected after reversal. There was a similar finding with the other TEG parameters (Table 4).

Reversal of heparin with protamine resulted in a significant decrease in ACT to a level similar to that observed following anivamersen dosing (Table 5). PT values decreased only modestly after protamine administration. The similar ACT values observed post-reversal of pegnivacogin and heparin support the interpretation that elevated coagulation measures post-reversal are due to haemodilution.

Thrombotic assessments

CPB was uneventful in all animals independent of treatment, with all CPB runs going to planned completion of 2 h on CPB and with no clots observed in the circuit, oxygenator or reservoir, with one
exception. In one REG1 animal for which pericardial blood was recirculated back to the pump, a small clot was observed in the reservoir at 1 h on CPB, which did not grow over the second hour on CPB. Following this observation, pericardial blood was no longer recirculated to the reservoir. Among the six baboons that received 1.5 mg/kg of pegnivacogin, 2 demonstrated clots, 1 in the venous cannula and 1 in the arterial cannula. Among the 5 remaining baboons that received the higher dose of 2.5 mg/kg of pegnivacogin, 3 of them also exhibited clots in the arterial cannula. All these clots appeared concurrent with cannulation and remained localized. No similar clots were seen in the heparin-treated group.

**Bleeding and haemostasis assessments**

There was a trend towards reduced intraoperative blood loss in REG1-treated animals when compared with heparin-treated animals as the greatest blood loss was observed in the two heparin-treated animals for which such data were collected (Table 6). Measurements of blood loss following reversal, both postoperative blood loss and time to haemostasis (end of reversal to skin closure), showed similar numerical trends as intraoperative blood loss (Table 6).

**Pathology and histology**

The major macroscopic findings were focal ischaemic lesions in the kidneys of REG1-treated animals as well as renal cortical tubular necrosis, neither of which were observed in heparin-/protamine-treated animals. These lesions, ranging from minimal to severe, were present on the right and left kidneys of 10/11 and 6/11, REG1-treated animals. They were characterized by sharp margins, a triangular transcortical shape and pale colour. Macroscopic findings were confirmed by microscopic analysis with observations of arterial and arteriolar thrombosis. The average microscopic severity score for the observed kidney lesions is presented in Table 7. Based on these data, the renal cortical arterial/arteriolar thrombosis and cortical tubular necrosis were judged to be related to REG1-treatment, with a potential trend towards reduced severity at the 2.5 vs 1.5 mg/kg pegnivacogin dose. Immunochemical investigation further confirmed the presence of thrombus in arterioles and arteries of the kidney.

Arteriolar thrombosis was further found in the lung in animals from both the REG1- and heparin-/protamine-treated groups. Furthermore, single thrombi were found in the right atria of two heparin/protamine and in one REG1-treated animal. These findings were confirmed by immunochemical analysis. These sporadic observations and balance between treatment groups do not allow the establishment of a clear relationship between these lesions and either anticoagulant regimen.

Globally, immunochemical analysis did not reveal any evidence of microthrombosis in investigated tissues from any animal in the REG1 or heparin/protamine treatment groups.

**Inter-species coagulation analysis**

In the inter-species comparison of thrombin-generating capacity, human, pig and sheep blood displayed close patterns with regard to blockade of thrombin generation in response to given doses of pegnivacogin whereas, at those same doses, baboons were unexpectedly found to be still able to generate substantial concentration of thrombin (Fig. 1A and B).

**DISCUSSION**

The main findings of this study are that, in a non-human primate model, the REG1 system is efficacious in reducing intra- and post-CPB blood losses but caused microthrombi at the cannula placement sites and cortical kidney infarcts. These findings are likely attributable to the baboon-specific pattern of tissue factor (TF)-induced thrombin generation, and the pegnivacogin appeared inadequate to inhibit this pathway relative to heparin/protamine.

Since the early days of OHS, heparin-based anticoagulation and its reversal by protamine sulphate have been the gold standard for preventing clotting during CPB. Aside from side-effects, which may be life-threatening such as heparin-induced thrombocytopenia [8] or protamine-related allergic reactions [3], this regimen does not consistently prevent post-CPB coagulation abnormalities, which contribute to postoperative bleeding (the prevalence of which is 2.4% in the 528 279 patients undergoing coronary artery bypass grafting and included in the Society of Thoracic Surgeons National Cardiac Database [9]), blood transfusions (up to 70% of patients in some series) and the attendant worsening of patient outcomes [1, 10, 11]. Limitation of bleeding is still more challenging in high-risk subsets like patients under mechanical assist devices [12] or adults undergoing reoperation after previous repair of congenital heart diseases [13] who represent an increasing proportion of the contemporary surgical case load. Thus, there is clearly room for improving the management of the coagulation system during CPB [14].

Throughout the period of extracorporeal support, there is measurable thrombin in the patient and the circuit [15, 16]. TF

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**Table 6: Average severity score for kidney thrombosis/ necrosis**

<table>
<thead>
<tr>
<th>Region</th>
<th>Heparin</th>
<th>1.5 mg/kg pegnivacogin</th>
<th>2.5 mg/kg pegnivacogin</th>
</tr>
</thead>
<tbody>
<tr>
<td>Arterial/arteriolar thrombosis</td>
<td>0</td>
<td>1.5</td>
<td>0.6</td>
</tr>
<tr>
<td>Cortical tubular necrosis</td>
<td>0</td>
<td>2.2</td>
<td>1.2</td>
</tr>
</tbody>
</table>

**Table 7: Average severity score for haemorrhage**

<table>
<thead>
<tr>
<th>Region</th>
<th>Heparin</th>
<th>1.5 mg/kg pegnivacogin</th>
<th>2.5 mg/kg pegnivacogin</th>
</tr>
</thead>
<tbody>
<tr>
<td>Adrenal</td>
<td>0.3</td>
<td>0.2</td>
<td>0</td>
</tr>
<tr>
<td>Aorta</td>
<td>3</td>
<td>2.7</td>
<td>2.2</td>
</tr>
<tr>
<td>Brain</td>
<td>0.7</td>
<td>0.5</td>
<td>0</td>
</tr>
<tr>
<td>Heart</td>
<td>2.7</td>
<td>2.5</td>
<td>1.8</td>
</tr>
<tr>
<td>Kidney</td>
<td>0.7</td>
<td>0</td>
<td>0.6</td>
</tr>
<tr>
<td>Liver</td>
<td>0</td>
<td>0.5</td>
<td>0</td>
</tr>
<tr>
<td>Lung</td>
<td>0.3</td>
<td>1.2</td>
<td>1.4</td>
</tr>
</tbody>
</table>
has been suggested to be its primary source [17] with increased concentrations of soluble TF found in samples taken from the pericardium [18]. Increased concentrations of VIIa have also been measured in samples from the pericardium with correspondingly raised concentrations of prothrombin F1+2 and thrombin–antithrombin (TAT) complexes, [19] which have been shown to be inversely correlated with heparin concentrations.

In this context of OHS, the anti-FIXa REG1 system looks appealing because it can be titrated, easily monitored and reliably reversed. Independent of whether coagulation is initiated by contact with a foreign surface or TF, the FVIIIa/FIXa complex drives amplification at the factor Va/FXa step and the positive-feedback cycle driven by thrombin generation [20–23]. Furthermore, FVIIia/FIXa-catalysed activation of coagulation factor X appears to be the rate-limiting step for thrombin generation [20–23]. Thus, FIXa represents an ideal target for anticoagulation across a range of indications.

To date, REG1 has been investigated in six clinical studies (three Phase I and three Phase II) with a total of 623 patients exposed to the investigational agent during PCI. Treatment with pegnivacogin resulted in a dose-dependent increase in aPTT, while treatment with the reversal agent (anivamersen) rapidly and durably reversed the pharmacological activity of the anticoagulant. In addition, 10 sheep and 23 pigs underwent successful CPB for periods of 1–4 h on pegnivacogin doses ranging from 0.5 to 4.5 mg/kg, with and without vessel graft replacement, with no observations of clotting during cannula placement or infarcts upon autopsy (REGADO data on file, personal communication Steven Zelenkofske, Christopher Rusconi).

The present study was thus planned as confirmatory in a non-human primate species prior to the first use of REG1 during CPB in humans.

The doses of pegnivacogin tested, 1.5 and 2.5 mg/kg, generally performed as expected with respect to PK parameters. The degree of anticoagulation at first glance may seem inadequate as the observed ACT and aPTT results were much lower than what is typically seen during CPB using heparin. However, both doses achieved a level of anticoagulation consistent with complete inhibition of FIXa activity as measured in aPTT, ACT and TEG assays. Administration of anivamersen at a dose ratio to pegnivacogin of 0.5:1 and 1:1 resulted in a decrease of plasma concentrations of pegnivacogin to a negligible level, and simultaneously to a reduction in measures of anticoagulation which, however, did not return to predose baseline values probably because of the significant CPB-induced haemodilution. Heparin/protamine performed as intended per protocol with respect to pharmacodynamic assessments, with a reversal of anticoagulation after protamine administration similar to that observed in REG1-treated animals. The CPB circuits were not primed with blood, consequently there were likely a number of effects related to haemodilution. A large drop in fibrinogen was observed after initiation of CPB, most likely related to haemodilution. The similar changes observed in RBC, WBC and platelets are most consistent with this explanation. The increase in aPTT in the REG1 animals following initiation of CPB can also be attributed to haemodilution. The PT was unaffected by pegnivacogin administration, as expected by a direct FIXa inhibitor. However, the most likely explanation for the increase in PT upon initiation of CPB in the REG1 animals was haemodilution. Haemodilution is also the most likely explanation as to why the ACT and aPTT did not return to baseline values after reversal with anivamersen.

All CPB cases were uneventful, with no observations of clotting within the CPB circuit, oxygenator or reservoir when pericardial blood was not recirculated into the CPB circuit. Intra- and post-operative measurements of blood losses favoured REG1; however, the animals in the control group were too few to draw concrete conclusions. Of concern, thrombus was observed during cannula placement in several REG1-treated animals, with the thrombus remaining localized to the cannula insertion site. Likewise, cortical kidney infarcts were uniquely observed in the majority of REG1-treated animals and likely related to the thrombus observed during cannula placement. Several mechanistic hypotheses were raised to explain these findings. The first was that dosage had been inadequate to achieve effective trough drug levels but pharmacological data do not support this possibility as these coagulation measures and drug concentrations levels fell within the range previously shown to effectively eliminate FIXa activity. Another possibility could have been size differences of baboons when compared with sheep or pigs, and the resulting smaller cannulae and/or lower pump flow rates that had to be used in the former setting. However, we could not test this hypothesis with the current data set. We finally reasoned that the differences in outcomes might rather reflect species-specific differences in the coagulation system and then implemented an additional head-to-head bench study to compare the TF-mediated thrombin-generating capacity in the presence of complete FIXa inhibition in different species. These experiments clearly showed that baboons uniquely generate significant thrombin upon TF activation in the presence of pegnivacogin, whereas thrombin generation in human, pigs and sheep was significantly inhibited by pegnivacogin under the same conditions. As exogenous TF was added to the plasma from the various species in these experiments, this indicates that the inhibitory pathways, such as TFPI, that limit TF/FVIIa generated thrombin to the initiation phase in humans and other species, may be less active in the baboon. This observation suggests that the thrombi observed in REG1 baboons were likely due to a species-specific TF-generated clot formation in regions of high TF-exposure, such as cannulation sites. In contrast, thrombin generation mediated by the contact-pathway appears to respond similarly to FIXa inhibition in baboon, pigs, sheep and human as evidenced by the aPTT response to pegnivacogin. This observation provides a mechanistic explanation for the successful performance of CPB with respect to the circuit and oxygenator despite the microthrombotic events observed upon cannulation in the majority of REG1-treated animals. Indeed, there are species-specific responses to a haemostatic insult [24] and our findings are consistent with the observations previously made in a baboon vascular occlusion model where a Factor XI monoclonal antibody inhibitor was effective in reducing thrombus formation without an increase in D-dimer levels [25]. Our investigations have led us to the conclusion that this inter-species difference renders the baboon model inappropriate for such investigations, although we acknowledge that others may construe this as an advantage.

**LIMITATIONS**

A major limitation is the small number of animals studied overall, but we believe this was reasonable considering the species tested. We assessed the intensity of anticoagulation in these experiments with aPTT, PT <ACT and thromboelastography supplemented fibrinogen measurements. Additional markers of fibrin activity such as TAT complex, D-dimer or fibrinopeptide A may have provided...
additional support regarding the intensity of anticoagulation in these experiments. Furthermore, we did not directly test the inhibitory effects of pegnivacogin (or heparin) on the procoagulant activity of the pericardial fluid—this may have provided additional support for our findings of inter-species differences in the TF pathway. While reinfusion of shed mediastinal blood is commonly practised, many groups have chosen not to do this. In these baboon studies, the practice was abandoned after clot was observed in the reservoir in a single animal. We did not directly test the safety of the reinfusion of shed mediastinal blood in additional animals, although we suspect that the practice is unsafe.

**CONCLUSION**

Although the microthrombotic events (at cannulation sites and in the kidneys) associated with REG1 are probably related to the observation that FIX inhibition in baboons does not greatly inhibit TF-mediated thrombin generation, these findings raise a safety issue that could preclude the use of REG1 during clinical open heart operations. This concern is actually strengthened by the recent interruption (temporally coincident with the completion of the present study) of the REGULATE trial (NCT01848106) that had been designed to assess the efficacy of REG1 in preventing periprocedural ischaemic and bleeding complications in patients undergoing PCI. Even though the decision seems to have been made primarily on the basis of allergic adverse events, possibly related to the polyethylene glycol carrier to which the nucleic acid portion is conjugated, the permanent termination of what was supposed to be a pivotal trial makes it highly unlikely that REG1 will be used in a surgical setting either. Beyond these clinically relevant considerations, our data could suggest that baboons are a suboptimal model to assess factorspecific coagulation inhibitors. Alternatively, the amplified fibrin production observed in baboons may be seen as an advantage, and may better reflect the coagulation activation seen during CPB in humans.

**ACKNOWLEDGEMENTS**

We thank Claire Maelle Fovet for her technical support during the course of the animal experiments and Dominique Ponthier for her expert conduct of cardiopulmonary bypass in baboons.

**Funding**

This work was supported by Regado Biosciences, Inc., Basking Ridge, NJ, USA. Stephen Frenses is supported by the Bernard S. Goldman Chair in Cardiovascular Chair in Cardiovascular Surgery.

**Conflict of interest:** At the time of this study, Steven Zelenkofske and Christopher Rusconi were employees of Regado Biosciences, Inc., Basking Ridge, NJ, USA.

**REFERENCES**


