Coagulation factor XI vaccination: an alternative strategy to prevent thrombosis

C. ZHONG,* L. ZHANG,* L. CHEN,* L. DENG† and R. LI*‡
*State Key Laboratory of Microbial Metabolism and School of Life Sciences & Biotechnology, Shanghai Jiao Tong University; †Shanghai HyCharm Inc., and ‡Engineering Research Center of Cell & Therapeutic Antibody, Ministry of Education, Shanghai, China

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Essentials
- Coagulation Factor (F) XI is a safe target for the development of antithrombotics.
- We designed an antigen comprising the human FXI catalytic domain and diphtheria toxin T domain.
- Antigen immunization reduced plasma FXI activity by 54% and prevented thrombosis in mice.
- FXI vaccination can serve as an effective strategy for thrombosis prevention.

Summary. Background: Coagulation factor XI serves as a signal amplifier in the intrinsic coagulation pathway. Blockade of FXI by mAbs or small-molecule inhibitors inhibits thrombosis without causing severe bleeding, which is an inherent risk of currently available antithrombotic agents. Objectives: To design an FXI vaccine and assess its efficacy in inhibiting FXI activity and preventing thrombosis. Methods: An FXI antigen was generated by fusing the catalytic domain of human FXI to the C-terminus of the transmembrane domain of diphtheria toxin. The anti-FXI antibody response, plasma FXI activity and antithrombotic efficacy in mice immunized with the FXI antigen were examined. Results: The antigen elicited a significant antibody response against mouse FXI, and reduced the plasma FXI activity by 54.0% in mice. FXI vaccination markedly reduced the levels of coagulation and inflammation in a mouse model of inferior vena cava stenosis. Significant protective effects were also observed in mouse models of venous thrombosis and pulmonary embolism. Conclusions: Our data demonstrate that FXI vaccination can serve as an effective strategy for thrombosis prevention.

Keywords: antithrombotic; diphtheria toxin; factor XI; inflammation; vaccine.

Introduction
Thromboembolic diseases, such as myocardial infarction, stroke, and venous thromboembolism, are the leading causes of morbidity and mortality in high-income and middle-income countries [1]. Anticoagulation has been a mainstay for the treatment of such diseases for many years [2]. Currently available anticoagulants, such as low molecular weight heparin, vitamin K antagonists, and activated factor X and thrombin inhibitors, all target zymogens or proteases that are essential for hemostasis. Therefore, these drugs impose a high risk of substantial bleeding, including intracranial hemorrhage and gastrointestinal bleeding [3–5].

Gene knockout studies in mice and epidemiologic observations in humans have demonstrated that FXI, which plays a modest role in hemostasis [6–8], is an important factor in promoting thrombosis. Indeed, FXI is a safer target for the development of antithrombotics. Indeed, some strategies targeting FXI or its activated form, including mAbs and small-molecule inhibitors, have shown encouraging results in preclinical studies [6]. In particular, an FXI antisense oligonucleotide has completed phase II clinical trials, with positive results [9].

Traditional prophylactic vaccination against infectious diseases has saved millions of lives in the last century. More recently, therapeutic vaccines have been developed for the treatment or prevention of non-communicable diseases [10–12], which typically elicit neutralizing antibodies against endogenous proteins whose functions are critical in promoting disease. Examples include vaccines against CTLA4 for melanoma [13], TM4SF5 for colon cancer [14], and PCSK9 for hypercholesterolemia [15,16].
In the present study, we evaluated vaccination as an alternative strategy for FXI inhibition and thrombosis prevention. We designed a recombinant antigen by fusing the catalytic domain of human FXI to the transmembrane domain of diphtheria toxin (DTT), and examined the antibody response, plasma FXI activity and antithrombotic efficacy in mice. Our data demonstrate that FXI vaccine can be used as an effective prophylactic agent against thrombosis.

Materials and methods

Reagents

The DNA sequences encoding DTT (amino acids 202–378) and the catalytic domain of human FXI (hFXIc) (amino acids 363–607) were chemically synthesized by Shanghai Sijia Biotechnology, Shanghai, China, and cloned into pUC19 to generate vectors pUC19–DTT and pUC19–hFXIc, respectively. Primers were synthesized by GenScript Biotechnology (Nanjing, China). A DNA gel extraction kit and a plasmid miniprep kit were purchased from Axygen (New York, NY, USA). BL21 (DE3), a glutathione S-transferase (GST) affinity column and PreScission Protease were purchased from Shanghai Rongjun Biotechnology (Shanghai, China). Recombinant mouse FXI was purchased from R&D Systems (catalog number 123607) were chemically synthesized by Shanghai Sijia Biotechnology, Shanghai, China, and cloned into pUC19 to generate vectors pUC19–DTT and pUC19–hFXIc, respectively. Primers were synthesized by GenScript Biotechnology (Nanjing, China). A DNA gel extraction kit and a plasmid miniprep kit were purchased from Axygen (New York, NY, USA). BL21 (DE3), a glutathione S-transferase (GST) affinity column and PreScission Protease were purchased from Shanghai Rongjun Biotechnology (Shanghai, China). Recombinant mouse FXI was purchased from R&D Systems (catalog number 4556-SE-010; Minneapolis, MN, USA). All other chemicals were purchased from local companies unless otherwise stated.

Construction of expression vectors

To construct the DTT–hFXIc expression vector, the DNA sequence of DTT–hFXIc was generated by overlapping PCR. Briefly, the DTT and hFXIc fragments were PCR-amplified from pUC19–DTT with primers P1 and P2, and from pUC19–hFXIc with primers P3 and P4. The PCR products were mixed and amplified with primers P1 and P4 to generate a DTT–hFXIc fragment. There is no linker between DTT and hFXIc in the expression construct of the DTT–hFXIc protein. To construct the DTT expression vector, the DTT fragment was PCR-amplified from pUC19–DTT with primers P1 and P5. All DNA fragments were digested with both BamHI and XhoI, and cloned into pGEX-6p-1. The primer sequences are listed in Table 1.

### Table 1

| Primer | Sequence (5’ to 3’) |
|--------|---------------------|
| P1     | CGC GGA TCC GAT GAT GAT GAT AAG ATA AAT CTT GAT TGG GAT GTC |
| P2     | ACG ATC CTG GGC TTG ATT TTG GTG GTG GGA CTA TTA TAG AAA TTA TGA ACT A |
| P3     | TAG TTC ATA ATT CTA AAT GTC CCA CCA CCA AAA TCA AGC CCA GGA TCG T |
| P4     | CCG CTC GAG CTA GAG CTA GGG ACG ATT ATA CGA ATT ATG |
| P5     | CCG CTC GAG CTA PPP ACG ATT ATA CGA ATT ATG |

Protein expression

The expression vectors pGEX-6p-1–DTT and pGEX-6p-1–DTT–hFXIc were transformed into BL21 (DE3), separately. A single colony was selected to start the culture in 50 mL of LB medium at 37 °C. Bacteria were grown in 8 L of LB medium at 37 °C until the OD_{600 nm} reached 0.6–0.8. Protein expression was then induced with 0.1 mM isopropyl-β-D-thiogalactoside at 16 °C for 24 h, and cells were harvested by centrifugation at 6000 × g for 5 min at 4 °C.

GST affinity chromatography

Cell pellets were resuspended in phosphate-buffered saline (PBS) (140 mM NaCl, 2.7 mM KCl, 10 mM Na$_2$HPO$_4$, 1.8 mM KH$_2$PO$_4$, pH 7.4) and sonicated (60 cycles of 5 s on ice). After centrifugation (12000 × g for 30 min at 4 °C), the supernatant was loaded onto GST affinity columns, according to the manufacturer’s instructions. The GST tag was removed by PreScission protease cleavage (4 °C, 20 h, in 50 mM Tris-HCl, 140 mM NaCl, 1 mM dithiothreitol, and 1 mM EDTA, pH 7.4), and this was followed by GST-tag affinity chromatography. Proteins were further purified with a Superdex G75 column (GE Healthcare, Piscataway, NJ, USA). The purified proteins were analyzed on 4–20% ExpressPlus PAGE gels (GenScript, Nanjing, China).

Animals

Male C57BL/6J mice were purchased from Vital River Laboratory Animal Technology (Beijing, China), and were maintained under specific pathogen-free conditions in Shanghai Jiao Tong University Animal Center. All animal studies were performed in accordance with institutional guidelines and with approval from the Institutional Animal Care and Use Committee of Shanghai Jiao Tong University.

Immunization of mice

Briefly, 100 μL of antigen was adsorbed onto 100 μL of 0.2% Alhydrogel (Brenntag Biosector, Frederikssund, Denmark). Then, male C57BL/6J mice aged 6–8 weeks were subcutaneously injected with 60 μg of antigens in 200 μL of the antigen-adjuvant preparation [17]. Two boosters were administered at 2-week intervals. Mice that received PBS were used to define baselines. To assess the...
efficacy of the vaccine, mice received DTT, and adjuvant served as a control. Blood samples were drawn from the mouse orbital sinus 1 week after the final booster, allowed to clot, and then centrifuged at 13 780 × g for 5 min to generate sera. The antibody response against mouse FXI was measured by ELISA. The ELISA was performed according to Leitner et al. [18]. In total, 100 ng of mouse FXI was coated on each well in a 96-well plate. Serum from the indicated mouse was diluted 1 : 100.

Activated partial thromboplastin time (APTT), prothrombin time (PT) and FXI activity assays

Briefly, 600 µL of blood was drawn by cardiac puncture under anesthesia, and mixed with 3.2% sodium citrate at a volume ratio (blood/sodium citrate) of 9 : 1. Then, the blood was centrifuged at 600 × g for 10 min at room temperature. Approximately 350 µL of plasma sample was loaded onto a Sysmex CA-550 coagulometer (TOA Medical Electronics, Kobe, Japan).

The APTT assay was initiated with Dade Actin Activated Cephaloplastin reagent (Siemens Healthcare Diagnostics) and calcium chloride [19]. The prothrombin time (PT) assay was initiated with Thromborel S (Siemens Healthcare Diagnostics, Marburg, Germany) [20]. Plasma samples from PBS-treated mice were pooled and used as a baseline reference. APTT and PT ratios were calculated by dividing the measured values by the corresponding baseline values.

For plasma FXI activity measurement, a plasma sample was diluted 12.5-fold with human FXI-deficient plasma (Siemens Healthcare Diagnostics) and subjected to APTT measurement. Pooled values from the PBS-treated mice were used as a reference.

Thrombosis induced by inferior vena cava (IVC) stenosis

IVC stenosis was performed according to Brill et al. [21]. Briefly, male C57BL/6J mice were anesthetized with intraperitoneal pentobarbital (50 mg kg⁻¹). The IVC was exposed, separated from the abdominal aorta, and bound to a 30-G needle with a 6-0 silk tie (Shanghai Pudong Jinhu Medical Products, Wuhan, China). After removal of the needle, two neurovascular surgical clips were applied at two positions below the ligation site for 20 s to induce endothelial damage. Twenty-four hours after induction, thrombi in the IVC were collected, fixed in 4% formalin for 24 h, photographed, and weighed.

Induction of pulmonary embolism

Thromboplastin (Thromborel S; Siemens Healthcare Diagnostics) was used to induce pulmonary embolism according to Ethan et al. [27]. Male C57BL/6J mice were anesthetized with intraperitoneal pentobarbital (50 mg kg⁻¹). Thromborel S was dissolved according to the manufacturer’s instructions, and diluted 40-fold with saline. Thromborel S was injected into each mouse in < 3 s at a dose of 7.5 µL g⁻¹. At this dose, ~ 25% of wild-type mice survived [27]. Given that 100% of mice challenged with thromboplastin develop dyspnea because of acute occlusion of pulmonary blood vessels [28], we evaluated the antithrombotic effect in this model by determining the time to the onset of dyspnea after infusion of thromboplastin. To quantify the number of thrombi in the pulmonary vasculature, mice were killed 3 min after thromboplastin injection. The lungs were removed, and fixed immediately in 4% formalin (pH 7.4) at 4 °C for 24 h. Tissues were dehydrated, embedded in paraffin, cut into 4-µm sections, and stained with Mayer’s hematoxylin and eosin. The average number of thrombi was determined from eight random fields (× 20) of the left lobe [29].

Statistical analysis

Data are presented as the means ± standard deviation. Comparisons were assessed with an unpaired two-tailed Student’s t-test. A P-value of < 0.05 was considered to be statistically significant.

Results

Antigen design, expression, and purification

Immunization with self-protein alone does not induce the production of autoantibodies [30]. Introducing foreign Th
epitopes into a self-molecule can help to break such immune self-tolerance [10,11]. DTT possesses three Th epitopes [31]. Therefore, it can potentially be used as a carrier protein for FXI vaccine design. Given that human antigens are effective at breaking immune tolerance in mice [16,32], we fused the catalytic domain of human FXI (79% identical to the mouse FXI catalytic domain in amino acid sequence) to the C-terminus of DTT to construct a recombinant antigen, DTT–hFXIc. The recombinant antigen was expressed in soluble form in Escherichia coli, and purified to homogeneity. In SDS-PAGE, DTT–hFXIc migrates at ~50 kDa, and DTT migrates at ~20 kDa (Fig. 1A).

**DTT–hFXIc immunization induces an anti-FXI antibody response and inhibits plasma FXI activity in mice**

An effective FXI immunogen should overcome immune tolerance and elicit the production of a specific anti-FXI antibody [10]. Thus, we first analyzed the antibody response in mice immunized with DTT–hFXIc. As shown in Fig. 1B, an antibody response against mouse FXI was observed in DTT–hFXIc-treated mouse sera but not in DTT-treated mouse sera, indicating that the designed antigen DTT–hFXIc can break immune tolerance to endogenous FXI. We next evaluated whether the antibody could inhibit the function of FXI in mice. Indeed, mice immunized with DTT–hFXIc showed a 54.0% ± 8.7% reduction in FXI activity in the plasma as compared with DTT-treated or PBS-treated mice (Fig. 1C). We further examined whether the reduction in FXI activity caused by DTT–hFXIc immunization has any effect on coagulation. The APTT assay was performed to measure the activity of the intrinsic pathway of coagulation. Consistent with the reduction in plasma FXI activity, the APTT ratio was increased by 22.4% ± 10.9% (Fig. 1D) as compared with control mice, indicating downregulation of the intrinsic coagulation pathway. The PT assay was also performed to measure the activity of the extrinsic pathway of coagulation. Figure 1E demonstrates that the PT ratio in DTT–hFXIc-treated mice remained similar to that in DTT-treated and PBS-treated mice, indicating that the extrinsic pathway was not perturbed by DTT–hFXIc vaccination.

**Fig. 1.** SDS-PAGE of antigens and inhibition of FXI activity in mice immunized with the antigens. (A) Antigens were loaded on 4–20% ExpressPlus PAGE gels under reducing conditions. Gels were stained with Coomassie Blue. Molecular mass markers are shown on the left. Lane 1: diphtheria toxin (DTT). Lane 2: DTT–human FXI catalytic domain (hFXIc). (B–E) Male C57BL/6J mice were immunized subcutaneously. One week after the third booster, the anti-mouse FXI responses were determined by ELISA with mouse sera diluted 1 : 100 (B). Plasma FXI activity (C), activated partial thromboplastin time (APTT) (D) ratio and prothrombin time (PT) (E) ratio were also measured. Each symbol represents one mouse (n = 6–8 per group). Mice with strikingly low OD values in the DTT–hFXIc group in (B) were considered to be non-responders, and were excluded from the FXI activity and APTT/PT assays. One sample in the APTT/PT assays failed, owing to instrument error. Comparisons were assessed with an unpaired two-tailed Student’s t-test: **P < 0.01, ***P < 0.001. PBS, phosphate-buffered saline.

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DTT–hFXIc vaccination reduces IVC stenosis-induced coagulation and inflammation in mice

Coagulation in the APTT assay is initiated by ellagic acid, which does not exist in the physiologic environment. To test whether DTT–hFXIc immunization has anticoagulation effects in vivo, mice were subjected to IVC stenosis, whereby coagulation is initiated by endogenous molecules.

The TAT plasma level was increased 2.5-fold and 2.2-fold, and the average circulating platelet count was decreased by 17.9% and 14.7%, in PBS-treated and DTT-treated mice, respectively (Fig. 2A,B), 6 h after IVC stenosis as compared with before IVC stenosis. These data reveal a procoagulation state in the early phase of thrombosis development [33]. In contrast, in DTT–hFXIc-treated mice, the TAT plasma level was increased 1.5-fold, which was a lower increase than that in PBS-treated and DTT-treated mice. The average circulating platelet count was negligibly reduced (0.2%), indicating that DTT–hFXIc immunization suppressed the coagulation response induced by IVC stenosis.

In addition to a procoagulant response, an elevated inflammation response is another hallmark of venous thrombosis in its early stage [33]. Indeed, in PBS-treated mice, IL-6 and MCP-1 levels increased 101.3-fold and 1.9-fold, respectively (Fig. 2C,D) 6 h after IVC stenosis. In addition, the circulating leukocyte count was decreased by 36.9% (Fig. 2E), indicating leukocyte consumption. DTT-treated mice showed slightly lower fold increases in plasma levels of IL-6 (31.1-fold) and MCP-1 (1.3-fold) than PBS-treated mice, and a similar percentage decrease in circulating leukocyte count (34.1%). In contrast, DTT–hFXIc-treated mice had a lower fold-increase in IL-6 plasma levels (12.9-fold) than DTT-treated mice, whereas MCP-1 plasma levels and circulating leukocyte counts were relatively unchanged. These data indicate that DTT–hFXIc immunization suppresses the inflammatory response induced by IVC stenosis.

**DTT–hFXIc inhibits thrombus formation in mouse models of thrombosis**

We further examined the antithrombotic effect of DTT–hFXIc vaccination by using two mouse models, the St Thomas model and the pulmonary thromboembolism model, which induce thrombosis by distinct mechanisms. In the St Thomas model, a combination of reduced blood
flow and endothelial damage in the IVC was applied to induce thrombosis [26]. Twenty-four hours after thrombosis induction, all of the PBS-treated mice developed thrombi that occluded the vessels and weighed $10.6 \pm 2.8 \text{ mg}$ (Fig. 3A,B). Similarly, DTT-treated mice developed thrombi with a weight of $8.7 \pm 4.4 \text{ mg}$, which was slightly but not significantly lower than the weight of thrombi in PBS-treated mice. In contrast, mice immunized with DTT–hFXIc showed a significant reduction in thrombus size, with a weight of $4.0 \pm 3.3 \text{ mg}$.

In the pulmonary thromboembolism model, thrombus was induced by infusion of thromboplastin into the IVC [26]. Although PBS-treated mice and DTT-treated mice developed dyspnea in 70–200 s (mean $124 \pm 48 \text{ s}$ for PBS-treated mice and $120 \pm 35 \text{ s}$ for DTT-treated mice) after infusion of thromboplastin, the time to the onset of dyspnea for DTT–hFXIc-treated mice was significantly longer ($189 \pm 42 \text{ s}$) (Fig. 4A). Consistently, histologic analysis of thrombus formation in lungs showed that, 3 min after infusion of thromboplastin, the number of thrombi varied among the three groups of mice (Fig. 4B). The thrombus numbers were $14.8 \pm 1.0$ per visual field ($\times 20$) for PBS-treated mice, $10.7 \pm 0.6$ for DTT-treated mice, and $4.2 \pm 1.3$ for DTT–hFXIc-treated mice (Fig. 4C), demonstrating a significant reduction in pulmonary thromboembolism after DTT–hFXIc vaccination.

**Discussion**

In this study, we have demonstrated that vaccination against FXI is an effective strategy for inhibiting plasma FXI activity in vivo and protecting mice from thrombosis. The recombinant antigen DTT–hFXIc contains hFXIc and three Th epitopes. The antigen elicited significant production of anti-mouse FXI antibodies, and reduced plasma FXI activity by $54.0\% \pm 8.7\%$. Furthermore, the APTT ratio was increased and systemic coagulation after IVC stenosis was decreased in DTT–hFXIc-treated mice. Our data suggest that a modest reduction in plasma FXI activity is sufficient to inhibit coagulation initiated both in vitro and in vivo, which is consistent with the result from the cynomolgus monkey study [34,35].

In addition to its role in hemostasis, FXI plays a role in the inflammation pathway [36–38]. Indeed, systemic inflammation was reduced in DTT–hFXIc-vaccinated mice after the induction of venous thrombosis. However, given that DTT immunization also decreased IL-6 and MCP-1 plasma levels, the inflammatory suppression by DTT–hFXIc vaccination may not be explained exclusively by the reduction in plasma FXI activity. Nevertheless, we hypothesize that the reduced inflammatory response in DTT–hFXIc-vaccinated mice may be associated with the suppression of thrombin generation, as evidenced by the downregulation of plasma TAT levels (Fig. 2A). Furthermore, von Bruhl et al. reported that the deposition of platelets in the IVC promotes leukocyte accumulation and neutrophil extracellular trap (NET) formation during deep vein thrombosis propagation. NETs, in turn, activate contact factors, including FXII, prekallikrein, and high molecular weight kininogen, thereby releasing the potent proinflammatory peptide bradykinin [33,39]. Although NETs and bradykinin were not evaluated in this study, the consumption of platelets and leukocytes was reduced in DTT–hFXIc-treated mice (Fig. 2B,E), which suggested reductions in platelet deposition and leukocyte accumulation. Thus, a limited level of inflammation caused by DTT–hFXIc vaccination may also be linked with the reduction in contact system activation.
The antithrombotic effect of DTT–hFXIc immunization was demonstrated in two mouse models of thrombosis. In the St Thomas model, mice immunized with DTT–hFXIc showed a significant reduction in thrombus size as compared with PBS-treated and DTT-treated mice. In the pulmonary thromboembolism model, the time to the onset of dyspnea was significantly increased, and the number of vessels that were obstructed with thrombi was reduced in DTT–hFXIc-treated mice as compared with PBS-treated and DTT-treated mice. DTT-hFXIc immunization resulted in a modest reduction in plasma FXI activity as compared with the maximum reduction achieved by FXI antisense oligonucleotide in primates [34,35] and humans [40]. Nevertheless, our result suggests that a 50% reduction in plasma FXI activity in mice is sufficient to inhibit thrombus formation, which is consistent with the observation made in baboons [34]. The modest reduction in FXI activity associated with DTT–hFXIc vaccination may be a result of antibodies induced by the catalytic domain of human FXI needing to cross-react with mouse FXI in vivo to function properly. Further investigations are warranted to determine how effective it would be to immunize humans with hFXIc.

Recently, a number of vaccines have been clinically tested for the treatment of chronic non-communicable diseases, such as hypertension, type II diabetes, and obesity [11]. One benefit of vaccination is that the efficacy can be sustained for months. For example, antibody responses induced by the IL-1β vaccine in type II diabetes patients can persist for 40 weeks [11]. The expected long-duration stability of FXI vaccine represents an important advantage for long-term anticoagulation therapy, because the administration frequency may be lower than a few times per year, which permits reasonable patient convenience and compliance.

It is noteworthy that there are some important concerns when vaccine is used to prevent thrombosis. First, the immune onset of vaccine requires a week or longer; it is therefore not suitable for emergency treatment. Vaccine is better suited for chronic indications, such as stroke prevention in atrial fibrillation patients and secondary prevention in patients with unprovoked venous thromboembolism [41]. Second, the efficacy of vaccines may vary with the strength of the individual’s immune response. In individuals with strong antibody responses, the plasma may be devoid of FXI activity, which may
lead to unexpected episodes of bleeding, especially when patients need to undergo emergency surgery or experience a major trauma. When such cases arise, a single dose of recombinant activated FVII followed by antifibrinolytic therapy may be an effective antidote for FXI vaccine during a bleeding crisis [42].

Another concern associated with DTT–hFXIc vaccination is related to potential off-target effect(s). As hFXIc has varying degrees of amino acid sequence similarity with other proteins, in particular proteins in the intrinsic coagulation pathway, such as prekallikrein (65% amino acid identity), FIX (38% amino acid identity), and FXII (35% amino acid identity), there is a possibility of cross-reactions. Further investigations are warranted to evaluate the target specificity of the vaccine and the side effects thereof.

In conclusion, we demonstrated that mice treated with an FXI vaccine showed reduced plasma FXI activity and thrombosis in an IVC stenosis model and a pulmonary thromboembolism model. Thus, vaccination against FXI is a promising strategy for thrombosis prevention.

Addendum
R. X. Li and C. H. Zhong conceived the experiments. C. H. Zhong, L. Zhang, and L. Chen performed experiments. C. H. Zhong, L. Deng, and R. X. Li wrote the manuscript.

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Disclosure of Conflict of Interests
The authors state that they have no conflict of interest. C. H. Zhong, R. X. Li and L. Zhang have patent WO2014/183649 A1 licensed.

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