Nonclinical evaluation of heparin binding copolymer – HBC1 for restoring of blood coagulation after heparin treatment

B. Kalaska[*], E. Sokolowska[*], K. Kaminski[**], J. Miklosz[*], Shin-ichi Yusa[***], A. Blazejczyk[****], J. Wietrzyk[****], I. Kasacka[*****], K. Szczubialka[**], D. Pawlak[*], M. Nowakowska[**] and A. Mogielnicki[*]

[*] Department of Pharmacodynamics, Medical University of Bialystok, Poland, amogiel@umb.edu.pl
[**] Faculty of Chemistry, Jagiellonian University, Krakow, Poland, szczubia@chemia.uj.edu.pl
[***] Department of Materials Science and Chemistry, University of Hyogo, Japan, yusa@eng.u-hyogo.ac.jp
[****] Department of Experimental Oncology, Polish Academy of Sciences, Wroclaw, Poland, wietrzyk@iitd.pan.wroc.pl
[*****] Department of Histology and Cytophysiology, Medical University of Bialystok, Poland, kasacka@umb.edu.pl

ABSTRACT

Protamine sulfate – a protein isolated from salmons is registered antidote of heparin. It is widely used to restore blood coagulation in case of severe bleedings related to heparin therapy, but may also induce serious side effects. We synthesized compounds able to bind heparin in blood. One of the Heparin-binding Copolymers (HBC) efficiently neutralized heparin activity in the living animal. HBC1 injected intravenously 3 minutes after heparin stopped bleeding and restored clotting to normal values. When administered in the therapeutic doses, HBC1 did not influence blood count and chemistry, platelets activity, cardiovascular and respiratory functions of rat. Intravital imaging revealed rapid elimination of polymer with urine. HBC1 is efficient, safe and easy to synthesize polymer that could replace protamine for the treatment of heparin associated bleeding. Our technology presents a pharmaceutical companies a unique opportunity to optimize a lead compound into a marketable therapeutic.

Keywords: heparin, antidote, polymer, thrombosis, coagulation, animal model

1 BACKGROUND

The incredible progress and wide use of various medical devices during intravascular or cardiac interventions increased the need for administration of anticoagulants, such as heparins. Protamine sulfate – a protein isolated from sperm of salmon fished around Japan is the only one registered antidote of unfractionated heparin (UFH). It is used more than 600,000 times each year in US alone to restore blood coagulation in case of severe bleeding related to heparin therapy after open-heart procedures. However, around one thousand deaths a year could be attributed to complications after protamine injection; mostly because of its allergenic properties. The quest to replace protamine has a long history, and was recently described [1]. Our group took a challenge 5 years ago. We have already shown some promising natural and synthetic polymers able to bind UFH [2-5]. Some of them have patents as heparin binding agents [6-8] with possible application in anemia [9]. Our present aims were to: synthesize new heparin-binding copolymers, select the most potent and safe one in vitro and show the efficacy and safety of most promising lead candidate for human medicine in preclinical studies.

2 MATERIAL AND METHODS

We synthesized a group of heparin binding copolymers (HBC). To estimate pharmacokinetics fluorescently labeled with Alexa Fluor 750 variants of the polymers were also obtained. Physicochemical characteristics of the polymers was performed to find out their molecular weight and dispersity of molecular weight. These polymers were tested for the ability to bind UFH in vitro. The amount of each polymer needed to bind 1 mg of UFH was determined using Azure A assay. The size of the particles formed in solution due to complexation of UFH with the polymers was found using dynamic light scattering (DLS) experiments. For the most promising polymers their interaction with serum proteins was also studied using gel electrophoresis and DLS. Blood count and aggregation in whole blood (Aggregometer Model 700, Chrono-log, USA) were performed to check potential blood toxicity. From several HBC polymers we selected *in vitro* the one – HBC1 with the best efficacy/toxicity index, and this lead polymer was further tested in 80 Wistar male rats, weighing 180-220 g and 30 NMRI-Foxn1nu/Foxn1nu mice weighing 20-30 g. All the procedures involving animals and their care were approved by Local Ethical Committees. They were randomly divided into 3 groups treated intravenously with PBS solution of: vehicle, UFH (300 UI/kg) and UFH+HBC1 (UFH 300 UI/kg followed by HBC1 1.95 mg/kg). We examined the efficacy of HBC1 in the model of arterial thrombosis electrically induced in the carotid artery of 36 rats according to experimental plan: UFH-3 min-

HBC1-7 min-electrical stimulus (1 mA)-10 min-thrombosis development-45 min-end. At the end tail bleeding time was measured, formed thrombus was removed (it was weighed after 24 h incubation) and blood from the heart for further measurement was collected. All the technical details were previously published by our team [3-4]. Additionally, 10 rats were injected with enoxaparin or enoxaparin followed by 5-minute intravenous infusion of HBC1. Changes in activated clotting time (ACT) were measured in blood samples collected from tail at the following time points: baseline, 5, 10, 30 and 60 minutes after anticoagulant administration. HBC1 infusion started at 5 minute of experiment and was continued for 5 minutes. The body distribution of HBC1 was measured in 0, 5, 30, 60 and 120 minute after the injection of UFH followed by AlexaFluor®750- labelled HBC1 to 30 mice (5 animals for each time point) using In-vivo MS FX PRO system (Carestream Health INC., USA). Then, brain, lungs, kidneys, heart, liver and spleen were isolated from mice and their fluorescence was measured ex vivo. Blood count and blood chemistry was monitored 1 hour or once a week in 28 rats for over 1 month. All organs were macroscopically examined for gross pathology. Samples of the lungs and liver were immediately fixed in formalin and routinely embedded in paraffin. Specimens were stained with hematoxylin and eosin for general histological examination. Additionally, we examined a possible acute toxicity of HBC1 injected intravenously to 15 heparinized rats in 3 times higher than therapeutic dose by monitoring blood pressure for over 1 hour (Plugsys, Transonics System, USA), heart rate, tissue perfusion, blood oxygen saturation, respiration rate, peak exhaled CO_2 and body temperature (PhysioSuite, USA).

3 RESULTS

3.1 In vitro tests

From a series of polymers obtained we selected the HBC1 polymer which exhibited the strongest ability to bind UFH (measured as the weight required to bind a unit mass of UFH), while the size of its complexes with UFH was the smallest. In vitro assays showed that only few polymers, including HBC1, did not change RBC count, platelet count and aggregation. Additionally, in vitro studies revealed the most suitable ratio of HBC1/heparin for in vivo studies.

3.1 Animal tests

UFH administered intravenously decreased thrombus weight, prolonged aPTT, bleeding time and increased anti-factor Xa activity. HBC1 reversed UFH effects to the control values (Fig. 1). Following the administration of the enoxaparin we observed significant rise in the ACT. Infusion of HBC1 rapidly shortened ACT value to normal levels and the neutralization was maintained throughout the course of the experiment (Fig. 2).

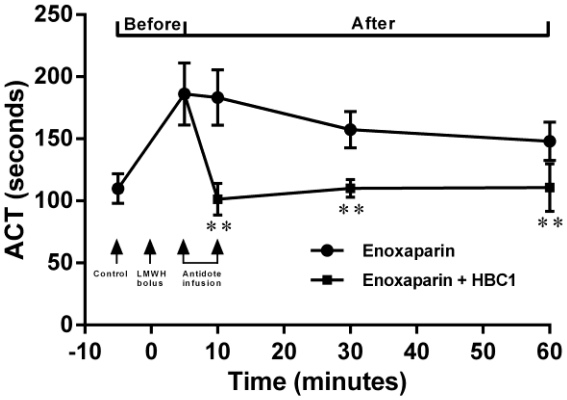

Figure 2: Reversing of ACT prolongation after enoxaparin by HBC1 in rats. ** P<0.01 vs. enoxaparin; Mann-Whitney test. Results are shown as mean ± SD, n = 6.

The signal of overall fluorescence was the highest 15 minutes after injection of Alexa Fluor®750-labeled HBC1 and could be detected in urine in all mice (Fig. 3). Ex vivo visualization of the fluorescent probe localized in isolated internal organs revealed that HBC1 is transiently distributed to liver (41% of the total fluorescence) and kidneys (18% of total fluorescence recorded). We did not observe any signal over 120 minutes in brain, lungs, heart and spleen. Although we found small pulmonary congestion in microscopic examination, none of the respiratory parameters changed within 1 hour after UFH+HBC1 administration.

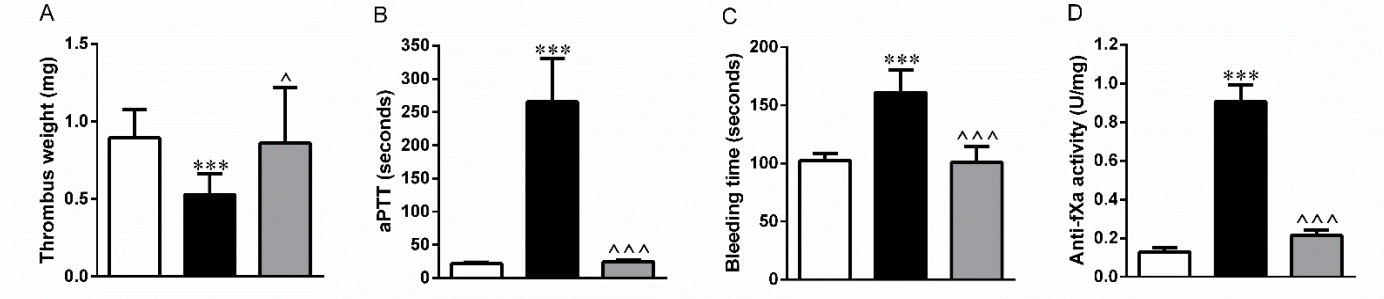

Figure 1. Reversing of UFH effects by HBC1 in rats developing arterial thrombosis.
*** P<0.001 vs. vehicle; ^ P<0.05, ^^^ P<0.001 vs. UFH 300 UI/kg, Mann-Whitney test.
Results are shown as mean ± SD, n = 9-14.

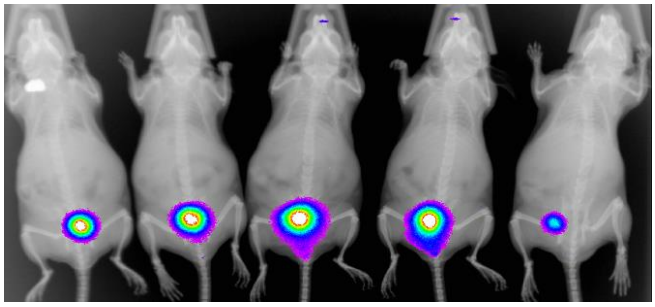

Figure 3: Distribution of HBC1 15 minutes after injection.

Routine histopathological examinations showed slight enlargement of hepatic cells 1 month after HBC1 injection. However, measurements in blood collected from rats once a week during 1 month did not reveal any hepatic, nephrotoxicity or blood abnormalities. HBC1 didn't change significantly blood pressure, heart rate and respiratory parameters, when infused into rats in efficient neutralizing dose.

4 CONCLUSION

HBC1 is easy to synthesize, efficient and safe heparin-binding copolymer that could replace protamine for the treatment and prevention of heparin associated bleeding. Significant restoring of Anti-factor Xa activity in heparinized animals and neutralization of anticoagulant effects of enoxaparin suggest good efficacy against low molecular weight heparins and fondaparinux, in case of which there are currently no specific antidotes. This property would extend the indications for use of HBC1, but needs financial support and further investigation. Our technology presents pharmaceutical companies unique opportunity to optimize a lead compound into marketable therapeutic.

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REFERENCES

[1] Bromfield SM, Wilde E, Smith DK (2013), Chem Soc Rev 42: 9184-9195.
[2] Kaminski K, Szczubialka K, Zazakowny K, Lach R, Nowakowska M (2010), J Med Chem 53: 4141-4147.
[3] Kaminski K, Plonka M, Ciejka J, Szczubialka K, Nowakowska M, Lorkowska B, Korbut R, Lach R (2011), J Med Chem 54: 6586-6596.
[4] Kalaska B, Sokolowska E, Kaminski K, Szczubialka K, Kramkowski K, Mogielnicki A, Nowakowska M, Buczko W (2012), Eur J Pharmacol 5: 81-89.
[5] Kaminski K, Kalaska B, Koczurkiewicz P, Michalik M, Szczubialka K, Mogielnicki A, Buczko W, Nowakowska M (2014), MedChemComm 5: 489-495.
[6] WO 2010002281 A1: Kaminski K, Zazakowny K, Szczubialka K, Nowakowska M (2009)
[7] US 20110301120 A1: Nowakowska M, Szczubialka K, Kaminski K, Zazakowny K (2011),
[8] US 20130034516 A1: Nowakowska M, Szczubialka K, Kaminski K (2013),
[9] WO 2013157967 A1: Nowakowska M, Szczubialka K, Kaminski K, Buczko W, Mogielnicki A, Kalaska B (2013)