# Dex40-GTMAC3 modified dextran as a novel heparin antagonist

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## ABSTRACT

Protamine is the only registered antidote preventing bleeding in patients treated with unfractionated heparin (UFH). However, protamine may induce anaphylactic shock or serious hypotension. We aimed to develop an alternative UFH antidote as efficient as protamine, but safer and easier to produce. As a starting material, we have chosen generally non-toxic, biocompatible, widely available, inexpensive, and easy to functionalize polymer - dextran. Our approach was to synthesize and purify dextran substituted with glycidyltrimethylammonium chloride (GTMAC) groups at a ratio of 0.65 per glucose unit (Dex40-GTMAC3), then to check it for potential heparin-reversal activity in vitro, and finally to examine efficacy and safety of the modified dextran in Wistar rats and BALB/c mouse models of experimentally induced arterial and venous thrombosis. Efficacy studies included the measurement of thrombus formation and coagulation parameters; safety studies included the measurement of pharmacokinetics, hemodynamic, hematologic, biochemical and immunogenic endpoints. Dex40-GTMAC3 is immediately eliminated from blood. It is as efficient as protamine but less immunogenic UFH inhibitor. Basing on our non-clinical evaluation of Dex40-GTMAC3, we believe that the novel polymer has very promising properties and can potentially substitute protamine as the UFH antidote.

***Keywords***: heparin, protamine, antidote, dextran, polysaccharides

## 1 BACKGROUND

Protamine is the only registered antidote preventing bleeding of patients treated with unfractionated heparin (UFH). However protamine, isolated from salmon sperm, may produce a number of adverse effects, such as anaphylactic shock or serious hypotension. We aimed to develop an alternative, safer than protamine, UFH antidote. This issue is continuously studied over many years but remains unsolved [1]. As a starting material, we have chosen generally non-toxic, biocompatible, widely available, inexpensive, and easy to functionalize polymer – dextran. We have already shown the potential of modified dextran to reverse heparin activity *in vitro* [2] and *in vivo* [3]. Our material and its indication for use to neutralize heparin activity or prevent anemia is worldwide patented technology [4,5].

## 2 METHODS

Basing on *in vitro* heparin-reversal activity assay of several polysaccharide cationic polymers we chose a high molecular weight dextran substituted with glycidyltrimethylammonium chloride groups at a ratio of 0.65 per glucose unit (Dex40-GTMAC3). The ability of Dex40-GTMAC3 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 polymer was found using dynamic light scattering (DLS) experiments. Interaction with serum proteins was also studied using gel electrophoresis and DLS. The potential direct blood toxicity of Dex40-GTMAC3 was estimated after incubation of polymer with whole blood by measuring: RBC, hemoglobin, hematocrit, mean corpuscular volume, hemoglobin and platelets in ABC Vet counter (Horiba, Germany) and osmotic resistance. The efficacy of Dex40-GTMAC3 was tested in 68 male Wistar rats weighing 217.7 ± 10.5 g, developing electrically induced arterial thrombosis [6], and 25 male BALB/c mice weighing 24.2 ± 0.2 g, developing electrolytic inferior vena cava thrombosis [7]. The pharmacokinetics, acute and chronic toxicity were estimated in 45 healthy male Wistar rats, weighing 220-300 g. The immunization experiment was performed in 20 female BALB/c mice weighing 26.3 ± 0.5 g. Animals were randomly divided into treatment groups. They were treated intravenously with UFH (in doses of 150 or 300 U/kg), Dex40-GTMAC3 (depending on the experiment in doses of 2.5, 3.75, 7.5 or 22.5 mg/kg b.w.) or protamine (1.5, 3 or 9 mg/kg b.w.). The control group received vehicle (phosphate buffer solution, PBS). All the procedures involving animals and their care were approved by Local Ethical Committees in Bialystok and Krakow (Permit Numbers 28/2012 and 92/2012, respectively) and conducted in accordance with ARRIVE guidelines, directive 2010/63/EU and the national laws. The efficacy endpoints in animal models of thrombosis were: arterial (rats) or venous (mice) thrombus weight, tail bleeding time, activated partial thromboplastin time (aPTT) (automatically determined in coagulometer – Coag-Chrom 3003, Bio-ksel, Poland), anti-factor Xa

activity (measured in microplate reader – Dynex Tech., USA according to instructions of Sekisui Diagnostics, USA kit). All the procedural details were previously published by our team [3,8]. Plasma concentration of Dex40-GTMAC3 labelled with fluorescein was fluorometrically measured, and pharmacokinetic parameters were calculated by using PK Solver Excel add-in program [9]. The blood pressure, heart rate, blood count and blood chemistry was measured 1 hour after administration of studied compounds to rats. In chronic setting body weight, blood count and blood chemistry were monitored once a week over 1 month. Brain, lungs, kidneys, heart, liver, spleen, bladder, the large and small intestine were collected from rats 1 hour or 28 days after single dose injection. All organs were macroscopically examined for gross pathology. To compare immune response of Dex40-GTMAC3 and protamine, the heparinization/neutralization regimen was repeated 5 times, once every week (days 1, 8, 15, 22, and 29). Blood samples were collected from the tail vein of each mouse one day prior UFH administration and serum was isolated by centrifugation. One week after the last injection (day 36) all mice were sacrificed; final blood samples were collected and spleens of the animals were isolated for evaluation. The levels of antibodies specific to protamine and Dex40-GTMAC3 were evaluated using standard indirect ELISA.

# 3 RESULTS

## 3.1 In vitro UFH binding by Dex40-GTMAC3

The concentration of free UFH in the solution decreased with increasing concentration of Dex40-GTMAC3. The amount of polymer necessary for binding 90% of 1 mg of UFH was used as a measure of the binding efficiency. This value was used for the preliminary assessment of the dose of a cationic polymer required in biological tests.

## 3.2 Reversal of the effects of UFH on the arterial thrombosis in rats

UFH decreased the weight of arterial thrombus in rats. Dex40-GTMAC3 reversed antithrombotic effect of UFH (Figure 1). The polymer was administered in doses corresponding with the ratio of polymer/UFH estimated in the UFH binding assay. When administered alone Dex40-GTMAC3 did not influence thrombus weight (0.98 ± 0.25 mg) in comparison to control group (0.92 ± 0.17 mg).

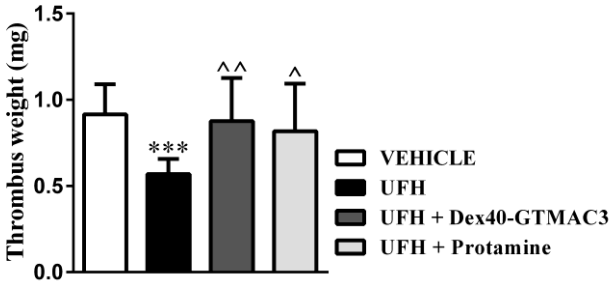


Figure 1: Reversal of the effects of UFH on the arterial thrombosis in rats. Dry thrombus weight in Wistar rats treated with vehicle (PBS), UFH (300 U· $kg^{-1}$) alone or followed by Dex40-GTMAC3 (7.5 mg·$kg^{-1}$), and protamine (3.0 mg·$kg^{-1}$), ***P<0.001 vs. vehicle; ^P<0.05, ^^P<0.01 vs. UFH 300 U·$kg^{-1}$, Mann-Whitney test. Results are shown as mean ± SD, n = 8-10.

## 3.3 Influence of Dex40-GTMAC3 on aPTT in rats

Dex40-GTMAC3 significantly shortened aPTT which was prolonged by earlier administration of UFH (Figure 2).

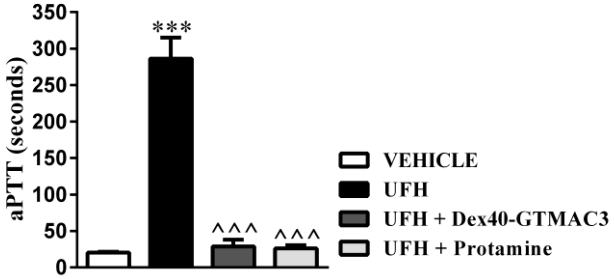


Figure 2: Influence of the cationic polymers on aPTT in rats. aPTT in Wistar rats treated with vehicle (PBS), UFH (300 U·$kg^{-1}$) alone or followed by Dex40-GTMAC3 (7.5 mg·$kg^{-1}$), and protamine (3.0 mg·$kg^{-1}$), ***P<0.001 vs. vehicle; ^^^P<0.001 vs. UFH 300 U·$kg^{-1}$, Mann-Whitney test. Results are shown as mean ± SD, n = 8-10.

## 3.4 Reversal of tail bleeding time by Dex40-GTMAC3

Similarly to aPTT measurement, UFH prolonged the tail transection bleeding time. When administered in the ratio of 2.5 mg for every 100 U·$kg^{-1}$ b.w of UFH, Dex40-GTMAC3 completely reversed tail bleeding time to the control value (Figure 3). Dex40-GTMAC3 administered alone did influence bleeding time (111.3 ± 18.9 sec.) in comparison to vehicle (104.3 ± 4.6 sec.).

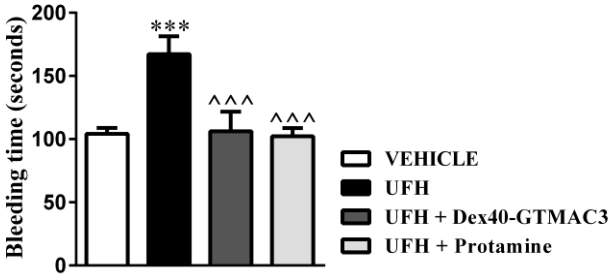


Figure 3: Reversal of tail bleeding time by the cationic polymers. Tail bleeding time in Wistar rats treated with vehicle (PBS), UFH (300 U·kg$^{-1}$) alone or followed by Dex40-GTMAC3 (7.5 mg·kg$^{-1}$), and protamine (3.0 mg·kg$^{-1}$), ***P<0.001 vs. vehicle; ^^^P<0.001 vs. UFH 300 U·kg$^{-1}$, Mann-Whitney test. Results are shown as mean ± SD, n = 8-10.

## 3.5 Reversal of plasma anti-Xa activity by Dex40-GTMAC3

Dex40-GTMAC3 significantly reversed increase of anti-Xa activity in UFH-treated animals (Figure 4).

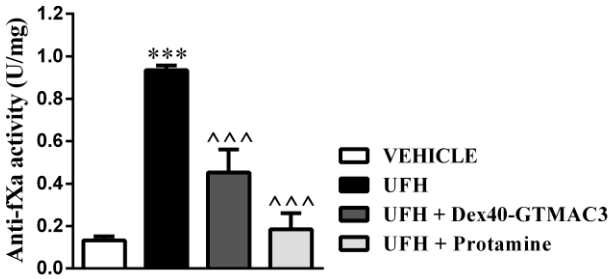


Figure 4: Reversal of plasma anti-fXa activity by the cationic polymers. Anti-fXa activity in Wistar rats treated with vehicle (PBS), UFH (300 U·kg$^{-1}$) alone or followed by Dex40-GTMAC3 (7.5 mg·kg$^{-1}$), and protamine (3.0 mg·kg$^{-1}$), ***P<0.001 vs. vehicle; ^^^P<0.001 vs. UFH 300 U·kg$^{-1}$, Mann-Whitney test. Results are shown as mean ± SD, n = 8-10.

## 3.6 Reversal of the effects of UFH on the venous thrombosis in mice

Similarly to the results in the model of the arterial thrombosis in rats, UFH significantly decreased thrombus weight and prolonged aPTT in mice developing electrically induced venous thrombosis. We confirmed the ability of Dex40-GTMAC3 to reverse antithrombotic (Figure 5) and anticoagulant (Figure 6) effects of UFH in mice.

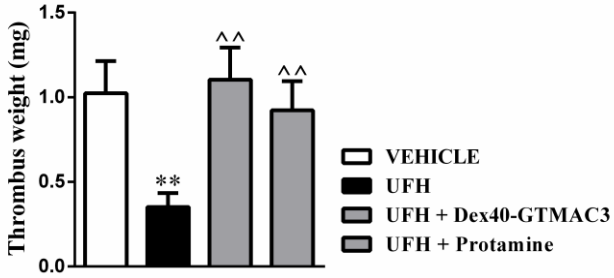


Figure 5: Reversal of the effects of UFH on the venous thrombosis in mice. Dry thrombus weight in mice treated with vehicle (PBS), UFH (300 U·kg$^{-1}$) alone or followed by Dex40-GTMAC3 (7.5 mg·kg$^{-1}$), and protamine (3.0 mg·kg$^{-1}$), **P<0.01 vs. vehicle; ^^P<0.01 vs. UFH 300 U·kg$^{-1}$, Mann-Whitney test. Results are shown as mean ± SD, n = 5-7.

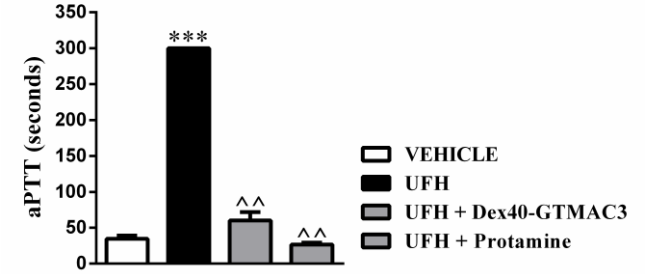


Figure 6: Reversal of the effects of UFH on aPTT in mice. aPTT in mice treated with vehicle (PBS), UFH (300 U·kg$^{-1}$) alone or followed by Dex40-GTMAC3 (7.5 mg·kg$^{-1}$), and protamine (3.0 mg·kg$^{-1}$), ***P<0.001 vs. vehicle; ^^P<0.01 vs. UFH 300 U·kg$^{-1}$, Mann-Whitney test. Results are shown as mean ± SD, n = 5-7.

## 3.7 Effects of Dex40-GTMAC3 on blood pressure in rats

We found significant, but much weaker and temporary change of mean blood pressure (MBP) shortly after administration of Dex40-GTMAC3 (decrease in MBP). If UFH was injected 3 minutes before the administration of Dex40-GTMAC3, the cationic polymer did not decrease MBP.

## 3.8 Evaluation of the immune response to Dex40-GTMAC3

IgG-class response was detectable in all mice in the protamine group beginning from day 21$^{st}$ on. On day 36 the mean protamine-specific IgG signal in the protamine group was significantly higher than the background signal in the UFH group (P<0.01). In contrast to this result, no significant levels of IgG specific to Dex40-GTMAC3 were detected (Figure 7).

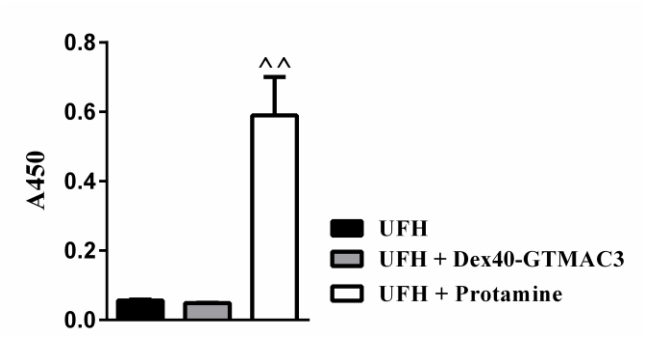


Figure 7: The levels of IgG specific toward Dex40-GTMAC3 or protamine in mice in 36 day of therapy presented as values of absorbance in ELISA test. ^^ P<0.01 vs. UFH, Mann-Whitney test. Results are shown as mean ± SD, n = 5.

### 3.9 Pharmacokinetic parameters, blood chemistry and toxicity studies

The concentration of Dex40-GTMAC3 in plasma after drug administration rapidly declined with $T_{1/2}$ of 29.4 ± 0.47 min. After 1 hour the amount of the circulating polymer was 7.7 % of the amount measured after 5 minutes. The toxicity studies did not reveal any pathological signs of acute or chronic hepato- or nephrotoxicity. The body weight, gross changes of internal organs, and blood chemistry remained the same as control values.

### 3.10 Blood count

UFH significantly increased white blood cells (WBC). Dex40-GTMAC3 partially, but significantly, inhibited increase of WBC in UFH treated animals. Dex40-GTMAC3 did not change any other blood count.

## 4 CONCLUSIONS

Documented efficacy without hepato-, nephro-, immunotoxicity or blood toxicity of Dex40-GTMAC3 make this agent advantageous over protamine. Dex40-GTMAC is easy to synthesize, efficient and safe heparin-binding polysaccharide polymer with a potential as marketable therapeutic.

## FUNDING

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