Published in final edited form as:

Thromb Res. 2011 October ; 128(4): e9–e13. doi:10.1016/j.thromres.2011.03.021.

Engineered virus-like nanoparticles reverse heparin anticoagulation more consistently than protamine in plasma from heparin-treated patients

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Abstract

Heparin is widely used for anticoagulation, often requiring the subsequent administration of a reversal agent. The only approved reversal agent for heparin is protamine sulfate, which induces well described adverse reactions in patients. Previously we reported a novel class of heparin antagonists based on the bacteriophage Q β platform, displaying polyvalent cationic motifs which bind with high affinity to heparin. Here we report heparin reversal by the most effective of these virus-like particles (VLP) in samples from patients who were administered heparin during cardiac procedures or therapeutically for treatment of various thrombotic conditions. The VLP consistently reversed heparin in these samples, including those from patients that received high doses of heparin, with greater efficiency than a negative control VLP and with significantly less variability than protamine sulfate. These results provide the first step towards validation of heparin antagonist VLPs as viable alternatives to protamine.

Keywords

heparin; anticoagulation; bacteriophage Q β ; protamine; coronary intervention; venous thrombosis

Unfractionated heparin (UFH) has been used as an anticoagulant since the 1940s. Despite its ubiquity in clinical settings, bleeding is a common and serious complication of heparin therapy [1,2]. Therapeutic UFH is a naturally occurring glycosaminoglycan that is purified from pig or bovine tissue as a heterogeneous mixture of polysaccharide chains with variable molecular weight (5–30 kDa, average 15 kDa) [3,4]. The anticoagulant function of heparin

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is due primarily to activation of the plasma serine protease inhibitor (serpin) antithrombin, which inactivates the coagulation serine proteases thrombin and factor Xa [5].

In cardiopulmonary bypass, UFH is given in high doses to prevent thrombosis in the patient or within the pump machine. At the end of the pump run, UFH is neutralized with protamine to allow normal coagulation. Protamine is the only FDA approved agent for the reversal of heparin anticoagulation, but adverse effects of protamine are significant and varied, including severe anaphylactic reactions and arterial hypertension [6,7]. Adverse reactions to protamine correlate with increased mortality and approximately 1900 additional deaths per year in the US can be attributed to these adverse events during cardiopulmonary bypass [8,9]. Consequently, there is a compelling scientific and pharmacologic rationale for developing alternative heparin reversal agents, and indeed several research groups have designed and tested new heparin antagonists [3,10–14]. Some of these appear to currently be under development by pharmaceutical companies.

We have previously described a unique heparin antagonist that takes advantage of the regular polyvalent structure of the protein capsid of bacteriophage Q β , an icosahedral RNA virus comprised of 180 copies of a 14.1 kDa coat protein. The Q β virus-like particles (VLPs) are 28 nm in diameter. Through molecular biological and chemical manipulation we engineered polyvalent displays of cationic motifs on this scaffold, producing VLPs that bound to and blocked the anticoagulant function of UFH in biochemical assays [15]. Here we investigate the clinical heparin reversal function of one of these active VLPs, in which the solvent-exposed residue Thr18 of the coat protein was mutated to Arg (T18R), thereby giving the particle substantially greater positive surface charge. The K16M mutant serves as a negative control since its positive charge is diminished relative to the wild-type structure and it did not bind or reverse heparin *in vitro* [15].

We collected plasma from patients who were treated with either therapeutic heparin or who were treated with high doses of heparin while undergoing cardiac catheterization procedures. Reversal of heparin anticoagulation in these patient plasma samples using the T18R and K16M particles was investigated, in comparison to protamine. The active polycationic particle showed significantly more consistent heparin reversal activity, with much greater potency in terms of particle concentration vs. protamine concentration.

Materials and Methods

Virus-like particles (VLPs) derived from bacteriophage Q β coat protein containing mutations of Thr18 to Arg (T18R) and of Lys16 to Met (K16M) were produced and isolated as previously described [15,16]. Purified particles were stored in 0.1 M HEPES, pH 7.4.

Citrated plasma samples were collected from patients treated with intravenous unfractionated heparin (UFH) at Scripps Green Hospital after informed consent was obtained. Patients were identified and screened through the cardiac catheterization laboratory and the Department of Pharmacy daily roster of patients treated with UFH. Samples were drawn at baseline prior to initiation of heparin, when feasible, and up to three consecutive plasma samples were drawn while the patient was receiving UFH. The clinical activated partial thromboplastin time (aPTT) and anti-Xa level, a clinical measure of heparin concentration, were determined for each sample. Patient anonymity was insured using an ID number randomization protocol. Demographic data was collected.

A total of 57 plasma samples were collected at different time points in 26 patients (Table 1). Diagnoses included cardiac arrhythmias, acute coronary syndrome, venous thromboembolism, and peripheral artery disease. Baseline samples drawn prior to UFH administration were collected for 6 patients who were undergoing cardiac procedures in the

cardiac catheterization laboratory. Then, following UFH administration, 1 to 3 samples were collected during the cardiac procedure, every 30 minutes. The remaining 20 patients were recruited from medical or surgical wards and were already on therapeutic UFH. Therefore, it was not possible to collect baseline samples for these patients. Plasma samples from therapeutic and high dose heparin treatment patient groups were studied separately.

For aPTT assays, VLP or protamine sulfate (MP Biomedical, Solon, OH, USA) was added to the patient plasma sample and incubated for 10 minutes at room temperature. APTT reagent (Triniclot HS, Trinity Biotech, Bray, Ireland) was then added and the reaction was incubated at 37 °C for 3 minutes before initiation of coagulation with CaCl₂. Clot time was measured with the Start4 viscosity-based detection system (Diagnostica Stago, Parsippany, NJ, USA). A heparin standard curve was generated by titrating UFH (Heparin, Baxter Healthcare, Deerfield, IL, USA) into pooled normal plasma (George King Biomedical, Overland Park, KS, USA) and assaying this titration with the same aPTT assay setup. With this standard curve aPTT times were converted directly to active heparin concentration remaining in the sample. Percent heparin reversal for each VLP-containing sample was then: (100 %) – (% heparin remaining).

For the factor Xa inactivation assay the Heptest kit (American Diagnostica, Stamford, CT, USA) was used. Protamine sulfate or VLP was added to the patient plasma sample and incubated for 10 minutes at room temperature before the assay was initiated. A standard curve was generated by adding UFH to pooled normal plasma and percent heparin reversal was determined in the same fashion as percent heparin reversal for the aPTT assay.

Statistical analysis

In Figure 1C data were fit to a straight line and the Pearson correlation coefficient (r) was calculated with GraphPad Prism v4.0 (La Jolla, CA, USA). The coefficient of determination (r^2) was presented. GraphPad Prism was also used to calculate mean and standard deviation of the data in Figs 2A and B for presentation in Fig. 2C and mean and standard deviation were also calculated for data in Fig. 3. For all data sets a Gaussian distribution was confirmed using GraphPad Prism.

Results

All of the therapeutic UFH samples were assayed for heparin reversal by the two VLPs using an aPTT assay. This assay was adapted for addition of VLP to the plasma samples, producing a reference clot time for pooled normal plasma of 46 seconds. Figures 1A and 1B show heparin reversal by T18R and K16M respectively for one plasma sample from each patient as a representative subset of all the data collected. T18R significantly reduced the clotting time in the aPTT assay, indicating that it was reversing heparin anticoagulant activity (Fig. 1A). In contrast, K16M produced a minimal effect (Fig. 1B).

Heparin reversal of all of the patient samples with 180 µg/mL added T18R or K16M was plotted in Fig. 1C. While some variability was apparent in the T18R results, the average correction was large and constant over the range of heparin concentration measured as anti-Xa units (69±10 % heparin reversal): in other words, T18R was effective over a wide range of therapeutic heparin plasma concentrations. The negative control K16M was far less effective (16±5 %). Duplicate experiments gave the same results for each individual plasma sample (paired t-test = 0.012). Heparin reversal by T18R showed a moderate positive correlation of percent reversal versus heparin concentration in the patient sample (coefficient of determination, r^2 = 0.35). There was no significant correlation for K16M.

The high dose UFH patient samples had clinical aPTT assay times >200 sec, which was the upper limit of the clinical assay. In our aPTT assay with buffer added rather than VLP, 13 of 14 high UFH samples did not clot at up to 1000 sec and one clotted at 750 sec (Fig. 2A, B; 0 points). This was in contrast to the therapeutic UFH samples in Fig. 1, of which the longest aPTT was 250 sec. The average baseline aPTT for control patient samples without added heparin or VLP (see Fig. 2D, 0 points) was 47.2 seconds (SD = 6.1, range = 40.2 to 57.3). With these samples we titrated either protamine sulfate or T18R VLP and monitored reduction in aPTT as an indicator of heparin reversal. Protamine sulfate reduced the aPTT in each patient sample, but in a highly variable manner (Fig. 2A). Some of the patient plasmas were reversed at low concentrations of protamine followed by subsequent prolongation of the aPTT at higher concentrations of protamine. Other patient plasmas were not corrected until four-fold higher concentrations of protamine were reached.

In contrast T18R generated a consistent pattern of heparin reversal in the patient plasma samples, with all reaching a minimum aPTT value of 58.5 sec (SD = 6.3, range = 46.0 to 67.4) (Fig. 2B). The difference in variability is illustrated in Fig. 2C, which shows the responses of individual plasmas from Figs 2A and 2B averaged together with standard deviations plotted. The extremely large standard deviations for protamine are contrasted with the much smaller standard deviations of T18R. A comparison of Figs. 2A and 2B shows that the reversal of heparin anticoagulation was achieved with less protamine compared to VLP in mass terms (approximately 11–44 µg/ml vs. 500 µg/ml, respectively, for complete reversal). However, the VLP was more efficient in terms of molar concentration of particles (approx. 2–9 µM protamine vs. 0.2 µM VLP; the coat protein molar concentration is 36 µM). It should also be noted that protamine is highly positively charged and contains a much higher percentage of positive charge than does the Qβ coat protein [15].

Titration into the control baseline plasma samples taken before administration of UFH showed T18R VLP to have minimal effect (slight prolongation of clotting time at the highest concentration, Fig. 2D). In contrast, protamine clearly caused significant prolongation of the aPTT in the absence of UFH and furthermore, this prolongation was highly variable depending on the plasma sample tested (Fig. 2D).

In these high-heparin samples, heparin reversal was also monitored in a clinical factor Xa inactivation assay. This assay differs from the aPTT in that it more narrowly monitors only factor Xa inactivation and therefore should not reflect nonspecific or general effects on the coagulation cascade. In this assay, both protamine and T18R VLP efficiently reversed heparin activity (Fig. 3A, B). Less variability in heparin reversal by protamine was observed here than in the aPTT assay, though at the lowest concentration of protamine the variability was still considerable (48 ± 16 % SD reversal). Significant variability was also found at the lowest concentration of T18R (36 ± 10 % SD reversal).

Discussion

The T18R VLP was found to reverse heparin anticoagulation at therapeutic levels significantly better than the negative control VLP, K16M (Fig. 1). Reversal was 69 ± 10 % with a slight increase in reversal as UFH concentration increased. The 10 % variability observed was remarkably low considering the expected differences in plasma from different patients. In a comparison of T18R to protamine sulfate with samples from patients treated with high doses of UFH during cardiac catheterization procedures, the VLP exhibited much more consistent results in aPTT clotting times (Fig. 2). Similarly, control samples from patients not treated without UFH were consistently unaffected by T18R but exhibited widely varying anticoagulation behavior when treated with protamine (Fig. 2D). Less difference in

the variability of UFH reversal by protamine sulfate and T18R VLP was observed in a clinical factor Xa inactivation assay (Fig. 3).

Prolongation of the aPTT clotting times as well as clotting times measured in other assays is a known effect of high doses of protamine [17,18]. This phenomenon is not well understood, but protamine was recently found to inhibit inactivation of factor IXa by antithrombin and to inhibit activation of factor Va [18,19]. Both of these findings were independent of heparin. In our study it is clear that the anticoagulant effect of higher dose protamine in the aPTT is quite variable from patient to patient both in the absence and the presence of heparin. The lack of an “anticoagulant” effect of protamine in the factor Xa inactivation assay (Fig. 3A) shows that the effect in the aPTT assay is not due to stimulation of the antithrombin reaction with factor Xa, which raises the possibility that the protamine anticoagulant effect in the aPTT may be an artifact. However, interactions of heparin with antithrombin and thrombin are significantly different and this assay does not address that interaction. Furthermore, the factor Xa inactivation assay does not address possible mechanisms of protamine anticoagulant activity that are independent of heparin. Nevertheless, in mice high dose protamine resulted in reduced thrombosis and prolonged tail bleeding times and in human plasma thrombin generation was inhibited [18], confirming that an anticoagulant effect of protamine overdose does happen in an *in vivo* mouse model as well as clinically. Since the aPTT is still used extensively in the hospital to monitor UFH and protamine during and after cardiac procedures [20,21], variability demonstrated in this assay is clinically relevant.

Although protamine dosing is determined by the amount and timing of UFH administration, several clinical studies have failed to demonstrate a correlation between UFH dose and the measured anti-Xa concentration or the patient’s activated clotting time (ACT) [22]. For this reason, and because of other adverse reactions, it is necessary to conservatively dose protamine proportionally to the expected amount of heparin in the patient and then continue to carefully monitor coagulation parameters and potentially adjust the dose of protamine further, as necessary [3,17]. A more consistent and safe alternative to protamine is therefore of great potential value.

This work does not address the mechanism of heparin reversal by cationic VLPs. We propose that binding is relatively non-specific as shown schematically in Figure 4 [15]. Protamine interaction with heparin is also relatively non-specific. Heparin binding of protamine peptide variants correlates closely with overall charge density rather than specific sequence [23,24]. We suggest that the polyvalency of positive charge on the T18R particle, and perhaps the particle’s size, is responsible for its high potency since the charge *density* of the virus coat proteins is much less than that of protamine. Of course, this non-specific binding may also result in unexpected interactions *in vivo*. Mechanism and *in vivo* function will be the focus of future studies.

Low molecular weight variants of protamine and synthetic variants of protamine have been reported to reverse heparin with lower toxicity than normal protamine [13,14]. However, in the 15 years since the first of these was described there have been no clinical trials of these molecules (clinicaltrials.gov). One potential limitation of these protamine-derived molecules is that LMW protamine appears to retain one significant shortcoming of protamine, which is susceptibility to heparin rebound [25]. Heparin rebound occurs because protamine has a very short circulatory half-life and UFH can be sequestered by binding non-specifically to many plasma proteins. Protamine binds to the free UFH and is then cleared allowing sequestered UFH to re-equilibrate into solution in the plasma [26]. We believe that heparin-antagonist virus-like nanoparticles may not be susceptible to heparin rebound because engineered VLP with positive charges have half-lives in mice on the order of hours [27] as opposed to protamine half-lives on the order of minutes [28].

This report expands our initial characterization of heparin antagonist VLP, which used pooled plasma with exogenously added UFH. We now demonstrate consistent function in individual plasma samples from patients treated with heparin. These results provide the first step towards validation of heparin antagonist VLP as a possible future alternative to protamine [29]. Such VLPs have several advantageous qualities that add to their promise for such applications. Studies of similar particles to those used here have demonstrated no toxicity, including in human vaccine trials [30–32]. However, heparin rebound and toxicity must be tested in future studies with these specific variants. In addition, VLP are easy to produce and purify in large quantities [16,27] and they are easily manipulated by both genetic and chemical means, which should make optimization of this first-generation heparin antagonist straightforward [33]. We will focus in the near future on such optimization, and on the detailed mechanistic characterization of the interaction between VLPs and various heparin species such as UFH and low molecular weight heparins.

Acknowledgments

This manuscript was supported by NIH/NCRR UL1 RR025774 (Pilot-Gale), The Stein Endowment Fund (Gale) and by NIH R01 CA112075 (Finn).

Abbreviations

UFH	unfractionated heparin
VLP	virus-like particle
aPTT	activated partial thromboplastin time

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29. One potential concern for therapeutic application of these VLP is immunogenicity. This concern will need further study, but is addressed by the following points. For acute administration, as in anticoagulant reversal in surgery, particle immunogenicity is irrelevant, since these particles are non-inflammatory and an immune response takes several days to mount. Furthermore, the circulation lifetime and tissue distribution for cowpea mosaic virus, a particle of very similar size as Q β , was the same in mice that were immunologically naïve as in those that had been previously

immunized with the particle (20). Similar results were seen with RGD-targeted M13 phage (24) suggesting that the activity of the particles may not be compromised by an immune response against them. If repeat administration is contemplated and the immune response is an issue, it may be possible to shield these particles from the immune system by attachment of poly(ethylene glycol) (PEG) or other immuno-shielding polymers (25). The key will be to do so without interfering with the binding abilities of the cationic domains, however, only a small fraction of the particle surface needs to be PEGylated to achieve a dramatic degree of shielding (26). If necessary, optimized heparin binding motifs can be translated to other particles that are either less immunogenic or that provide a different target for the human immune system.

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Figure 1. Heparin reversal by virus-like nanoparticles in patient plasmas with therapeutic heparin

A. Concentration dependence of T18R virus-like particle reversal of heparin-prolonged aPTT. B. Concentration dependence of K16M virus-like particle reversal of heparin-prolonged aPTT. C. Percent heparin reversal in patient plasmas. Virus-like particle was added at 180 $\mu\text{g/ml}$ in plasma, T18R (Δ), K16M ($\circ$).

Figure 2. Heparin reversal by virus-like nanoparticles in plasma from patients undergoing coronary intervention measured in an aPTT assay
 aPTT of plasma are presented after incubation with varying concentrations of protamine or T18R VLP. Plasma samples were collected from six patients before (control without heparin) and during (every 30 minutes) the coronary procedure. A. Protamine correction of aPTT prolongation of individual patient samples. B. T18R VLP correction of aPTT prolongation of individual patient samples. C. Average correction of aPTT prolongation of patient samples, protamine (■), n=14, T18R VLP (▲), n=12. Error bars = SD. D. aPTT of control patient samples without heparin, protamine (■), T18R VLP (▲). A reference aPTT value (47.2 sec) that is the average aPTT value for control patient plasmas without added heparin or VLP is indicated as a ● at X = 0 in panels A, B and C.

Figure 3. Heparin reversal by virus-like nanoparticles in plasma from patients undergoing coronary intervention measured in a Xa inactivation assay

Percent reversal of heparin in plasma samples are presented after incubation with varying concentrations of protamine or T18R VLP. A. Protamine reversal of heparin Xa inactivation activity of individual patient samples. B. T18R VLP reversal of heparin Xa inactivation activity of individual patient samples.

Figure 4. Schematic representation of cationic Q β particles interacting with anionic heparin polysaccharides

For reference, approximate molecular weights: 14.1 kDa for the virus coat protein, 14 kDa for heparin, 2.6 megaDa for the assembled virus particle.

Table 1**Clinical and Laboratory Characteristics of Heparin Treated Patients**

	Therapeutic Heparin	High Dose Heparin
Number of patients	20	6
Age (yr $\pm$ SD)	70.6 $\pm$ 12.0	66.2 $\pm$ 11.4
Male gender (n(%))	11(55)	4(66)
aPTT (sec $\pm$ SD)	80.5 $\pm$ 31.6	199.4 $\pm$ 2.2
Anti-Xa (units $\pm$ SD)	0.54 $\pm$ 0.24	2.1 $\pm$ 0.6
Clinical diagnosis	ACS, VTE, PAD, AF	VT, SVT

yr=years; SD=standard deviation; aPTT=measured in seconds, mean value over multiple time points, therapeutic heparin n=35, high heparin n=12; anti-Xa=measured in units, mean value over multiple time points, therapeutic heparin n=35, high heparin n=13; ACS=acute coronary syndrome; VTE=venous thromboembolism; PAD=peripheral artery disease; AF=atrial fibrillation; VT=ventricular fibrillation; SVT=supraventricular tachycardia.