Antibodies Against Lepirudin are Polyspecific and Recognise Epitopes on Bivalirudin

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Abstract

Bivalirudin is a synthetic anti-thrombin, sharing a sequence of 11 amino acids with the recombinant hirudin lepirudin. We investigated whether anti-lepirudin-antibodies recognize epitopes on bivalirudin.

Anti-lepirudin antibody positive sera of 43 patients, treated with lepirudin for heparin-induced thrombocytopenia, were analysed. Lepirudin- and bivalirudin coated microtiter plates were used for antibody testing in an ELISA system.

Of the 43 sera containing antibodies binding to lepirudin, 22 (51.2%) contained antibodies that also recognized bivalirudin. Binding of these antibodies to bivalirudin was inhibited by >70% by preincubation with high doses of bivalirudin. However, if lepirudin coated microtiter plates were used, high concentrations of bivalirudin inhibited only 2 of the 43 positive sera by >30%.

Therefore anti-hirudin antibodies must be polyspecific. The clinical consequences of this cross-reactivity are unknown but bivalirudin, targeted by antibodies of patients treated with lepirudin previously, could potentially boost antibody titres in such patients, or even trigger an immune response by itself. Clinically significant antibody formation in response to bivalirudin monotherapy has not been observed, however. Yet, as lepirudin and anti-lepirudin antibodies have recently been implicated in severe anaphylactic reactions, caution is warranted when using bivalirudin in patients previously treated with lepirudin.
Introduction

Bivalirudin is a small (MW 2180 Daltons) synthetic peptide modelled after hirudin and consists of 20 amino acids, containing two binding domains that bind directly and reversibly to the catalytic site and to the anion-binding exosite of thrombin (1,2). Bivalirudin is approved for use as an anticoagulant in patients with unstable angina undergoing percutaneous coronary interventions (3,4) and under investigation in patients with acute coronary syndromes (5-7), acute myocardial infarction (8-11), and in cardiac surgery patients with heparin induced thrombocytopenia (HIT). Bivalirudin has been successfully used as an alternative anticoagulant to treat patients with HIT (12,13).

Hirudins are proteins 65 amino acids in length and of non-human origin (medical leech). Anti-hirudin antibody formation in patients treated with recombinant hirudins has been demonstrated in several studies: in a prospective trial 87/198 (44%) HIT-patients treated with lepirudin in therapeutic dose developed anti-hirudin antibodies of the IgG class (14). In another study 74% of HIT patients developed anti-hirudin antibodies of the IgG-, IgA, and/or IgM-class (15,16). The risk of immunisation per time exposed seems to be in a similar range if desirudin, an r-hirudin similar to lepirudin, is given in prophylactic dose (desirudin 15mg s.c. bid) (17). Antibodies of patients treated with desirudin showed a 100% cross-reactivity to lepirudin and vice versa, and therefore must recognize structures common to both hirudins with similar immunogenicity (17). Recently lepirudin has been linked to at least nine cases of severe anaphylaxis, four of them with fatal outcome (18).

The amino-acid sequence hirudin and bivalirudin have in common binds to the exosite 1 of thrombin, as do other thrombin substrates. Clinically significant antibody formation in response to bivalirudin monotherapy seems to be low, but as
immunogenicity of lepirudin is high and lepirudin and bivalirudin share a sequence of 11 amino acids (Figure 1) we sought to determine the extent of in vitro cross-reactivity of anti-lepirudin antibodies with bivalirudin.
Methods

Study objective The objective was to assess whether anti-lepirudin antibodies in sera of lepirudin-treated patients recognize epitopes on bivalirudin.

Sera The study included sera from HIT patients treated with lepirudin who developed anti-lepirudin antibodies. Patients were treated with lepirudin in therapeutic (bolus followed by aPTT adjusted i.v. infusion; n=32) or prophylactic (aPTT adjusted i.v. infusion; n=11) doses. Mean treatment duration was 13.9 days. All sera were stored in aliquots at –30°C, thawed and tested.

Lepirudin (Refludan®, Schering AG, Berlin, Germany) as well as bivalirudin (The Medicines Company, Parsippany, USA) were obtained from the manufacturers.

Methods An ELISA for detection of anti-lepirudin IgG antibodies was performed as described before (14) with the following modifications:

Microtiter plate wells (high binding capacity, Greiner, Frickenhausen, Germany) were coated with either 10 µg/well lepirudin or 10 µg/well bivalirudin in coating buffer (8 mM NaH₂PO₄ x H₂O, 53 mM Na₂HPO₄, pH 7.5) overnight at 18-24° C, washed four times (0.15 m NaCl, 0.1% Tween 20, pH 7.5), incubated (60 min, 37°C) with sera of lepirudin-antibody positive patients (100 µl of 1/50 in sample dilution buffer [0.05 m NaH₂PO₄ x1H₂O, 0.15 m NaCl, 7.5% goat serum, pH 7.5]), washed four times, incubated with 100 µl goat anti-human IgG-HRP conjugate (Jackson, Hamburg, Germany; 60 min, 37°C, in sample dilution buffer), washed four times, incubated with 100 µl per well substrate solution (o-phenylenediamine dihydrochloride, DAKO, Glostrup, Denmark) (30 min, room temperature, in the dark). The reaction was stopped by adding 100 µl 0.5 M H₂SO₄ and optical densities (OD) were measured at 492 nm.
For inhibitory experiments sera were preincubated with high concentrations of either lepirudin (100 µg/ml and 400 µg/ml) or bivalirudin (1 mg/ml). As control, albumin was added in different concentrations (50, 100, 500, 1000 µg/ml).

Cut off values were defined based on serum samples from 100 healthy blood donors. Assay results were deemed negative or positive (99% quantile of blood donor data). All samples and standards were tested in duplicate.

OD values of binding experiments and of inhibitory experiments were compared by the Wilcoxon rank sum test.

Relevance of the antibodies in vivo In 28 of 43 patients lepirudin dose and aPTT levels during treatment were available. aPTT courses and dose changes were analyzed.
Results

A total of 43 sera of patients who had previously developed anti-lepirudin antibodies were assessed. In the lepirudin coated ELISA 43/43 (100%) samples gave a positive result.

21/43 sera (48.8%) contained antibodies that recognized lepirudin but not bivalirudin. These antibodies were inhibited by preincubation with high concentrations of lepirudin (400 µg/ml) but not by high concentrations of bivalirudin (1 mg/ml) (figure 2a).

22/43 sera (51.2%) contained antibodies which bound to lepirudin and to bivalirudin (figure 2b). When lepirudin was coated to microtiter plates a higher OD occurred as compared to bivalirudin coated microtiter plates. Antibody binding to lepirudin was inhibited dose dependently by lepirudin in the fluid phase (mean OD with buffer: 1.600 ± 0.193, mean OD with 100 µg/ml lepirudin added: 1.191 ± 0.404; mean OD with 400 µg/ml lepirudin added: 0.739 ± 0.480), whereas high concentrations of bivalirudin (1 mg/ml) had only a minor impact on antibody binding to lepirudin (mean OD with buffer: 1.600 ± 0.193 vs mean OD with 1 mg/ml bivalirudin added: 1.487 ± 0.290, p=0.227). When bivalirudin was coated, lepirudin in high concentrations inhibited antibody binding (mean OD with buffer: 0.969 ± 0.347 vs mean OD with 400 µg/ml lepirudin added: 0.231 ± 0.207, p<0.0001) as did high concentrations of bivalirudin (mean OD with buffer: 0.969 ± 0.347 vs mean OD with 1 mg/ml bivalirudin added: 0.076 ± 0.085, p<0.0001).

Albumin did not cause inhibition at any concentration tested (mean OD at 50 µg/ml 1.654 ± 0.119; 100 µg/ml 1.663 ± 0.124; 500 µg/ml 1.676 ±0.136; 1000 µg/ml 1.502 ± 0.141; buffer instead of albumin 1.600 ± 0.193). Each concentration was tested with 22 sera.
Of 14 evaluable patients with antibodies only reacting with lepirudin, in 8 there was a potentially enhancing effect of the antibodies i.e. aPTT increased from 57-80 sec to 71-159 sec while lepirudin dose was maintained (n=3) or aPTT was stable or even increased while lepirudin dose was reduced up to 62% (n=5). In 4 patients there was no obvious effect. In 2 patients there was a potentially inhibitory effect associated with the anti-lepirudin antibodies (in one patient the aPTT decreased while keeping lepirudin dose stable, in the other despite an increase in lepirudin dose the aPTT decreased).

Of 14 evaluable patients with antibodies cross-reacting with bivalirudin in 9 there was an enhancing effect, and in 5 no biological effect could be seen when aPTT and lepirudin dose changes were assessed.
Discussion

As bivalirudin and lepirudin have a segment of their respective amino acid sequences in common, cross-reactivity of anti-hirudin antibodies to this structurally similar site of bivalirudin is a plausible concept. Indeed, using sera of 43 well-characterised patients with anti-lepirudin antibodies, we confirmed this assumption: 22 sera (51.2%) contained antibodies binding to epitopes on bivalirudin (figure 2b). Binding specificity was demonstrated by inhibition of >70% by preincubation with free bivalirudin. As binding of antibodies to lepirudin was not inhibited by high concentrations of bivalirudin whereas binding to bivalirudin was inhibited by both high concentrations of lepirudin and high concentrations of bivalirudin, patient sera must contain polyspecific antibodies recognizing different epitopes on the lepirudin molecule, some of them shared with bivalirudin.

Generally lower antibody binding to bivalirudin as compared to lepirudin was probably caused by the fact that bivalirudin does not share all of the epitopes to which the polyspecific response to lepirudin is directed. Thus, not all anti-hirudin antibodies in a particular patient serum can actually bind bivalirudin. Steric inhibition occurring after binding of the small bivalirudin molecule to the microtiter plate and different coating characteristics may also decrease antibody binding.

Anti-hirudin antibody formation has been demonstrated in animal experiments (19,20), but only very rarely in healthy volunteers (21) or in patients with acute coronary syndromes treated with hirudins (22,23). Recently it became obvious that in patients treated with lepirudin for periods of more than 5 days, e.g. for treatment of heparin-induced thrombocytopenia, development of anti-hirudin antibodies is relatively common. These antibodies can be biologically relevant. As we observed previously (14), also in this study the most common effect of the anti-lepirudin
antibodies was enhancement of the anticoagulatory effect (17/28;61%) while inhibitory effects were much less frequent (2/28;7%).

Two patients with allergic reactions following re-exposure to the hirudins desirudin and lepirudin, respectively, have been reported (24,25). One of them was a volunteer who had received desirudin five years earlier and who developed urticaria after he was re-exposed twice with a one week interval between the subcutaneous injections (24). The second patient developed symptoms of an allergic response during re-exposure to lepirudin. Both patients tested positive for anti-hirudin antibodies (25).

Recently lepirudin has been linked to at least nine cases of severe anaphylaxis, four of them with fatal outcome. Most of the anaphylactic reactions occurred during re-exposure and in cardiac patients (18).

In contrast no clinically relevant anti-bivalirudin-antibody formation from any study including studies in which bivalirudin was given intravenously for at least 5 days has been reported to date. Among 494 patients who received bivalirudin in clinical trials and were tested for antibodies, only two had positive bivalirudin antibody tests. Neither patient demonstrated clinical evidence of allergic or anaphylactic reactions (26). In a study of 222 patients (27) who received bivalirudin by subcutaneous administration two to three times daily for up to 14 days, no antibody formation occurred up to 6 weeks (personal communication, Dr Teuber, The Medicines Company).

Our experiments lead to three important implications: firstly, in patients who have developed anti-lepirudin-antibodies, subsequent treatment with bivalirudin should be monitored carefully, because pharmacokinetic alterations might occur by reduced renal filtration of the bivalirudin-antibody complexes. As the shared structure of both molecules is the functional part, cross-reacting anti-hirudin antibodies should
preferably bind to these epitopes, potentially exerting additional functional effects, although we did not see a major difference in the biological effects between antibodies recognizing lepirudin alone and antibodies recognizing lepirudin and bivalirudin. The second conclusion, on a more speculative note, is that bivalirudin, sharing epitopes with lepirudin, might lead to boosting of anti-hirudin antibody titres, or even formation of anti-bivalirudin antibodies independent of any lepirudin treatment. Thirdly, and most importantly, anaphylaxis occurring in patients re-exposed to lepirudin, presumably due to anti-lepirudin antibodies, might also occur upon exposing such patients to bivalirudin and vice versa.

It is quite possible that the epitope generating the cross-reacting antibody may be different from those responsible for the severe immune responses upon re-exposure, given the multiple epitopes on lepirudin which trigger antibody formation and the relatively small homologous sequence between lepirudin and bivalirudin.

However, for safety reasons caution is warranted when bivalirudin is used in patients who have been treated with lepirudin or desirudin.
References


Figure Legends

Figure 1

Structural similarities between lepirudin (a) and bivalirudin (b). The sequence of 11 amino acids in the C-terminus (hatched) is identical in lepirudin and in bivalirudin. (The two-dimensional structure of the molecules is arbitrary.)

Figure 2 a,b

Sera of 43 patients who developed anti-hirudin antibodies during treatment with lepirudin were incubated with lepirudin or bivalirudin coated microtiter plates as described under methods. To demonstrate binding specificity, sera were preincubated with either buffer or lepirudin in high concentrations (400 µg/ml) or bivalirudin in high concentrations (1 mg/ml), respectively.

a) 21 sera contained antibodies which bound to lepirudin (lane 1) but not to bivalirudin (lane 4). Binding to lepirudin was strongly inhibited by high concentrations of lepirudin (lane 2) but only very weakly by high concentrations of bivalirudin (lane 3). Only 2 sera contained antibodies which binding to lepirudin was inhibited by >30% by high concentrations of bivalirudin.

b) 22/43 sera contained antibodies which bound to lepirudin (lane 1) and to bivalirudin (lane 4). When lepirudin was coated to microtiter plates a higher OD occurred as compared to bivalirudin coated microtiter plates. Antibody binding to lepirudin was inhibited by high concentrations of lepirudin (lane 2), whereas high concentrations of bivalirudin had only a minor impact on antibody binding to lepirudin (lane 3). Conversely, when bivalirudin was coated, lepirudin in high concentrations inhibited antibody binding (lane 5) to a similar extend as did high concentrations of bivalirudin (lane 6).
Figure 1

(a) lepirudin

(b) bivalirudin

binding to exosite 3 (active site) of thrombin

binding to exosite 1 (fibrinogen binding site) of thrombin
Figure 2a

The diagram shows the OD at 492 nm for different treatments:
- Lepirudin (10 µg/well)
- Lepirudin (10 µg/well)
- Lepirudin (10 µg/well)
- Bivalirudin (10 µg/well)
- Bivalirudin (10 µg/well)
- Bivalirudin (10 µg/well)

Coating of microtiter plate with:
- Lepirudin (10 µg/well)
- Lepirudin (10 µg/well)
- Lepirudin (10 µg/well)
- Bivalirudin (10 µg/well)
- Bivalirudin (10 µg/well)
- Bivalirudin (10 µg/well)

Preincubation of sera with:
- Buffer
- Lepirudin (400 µg/ml)
- Bivalirudin (1 mg/ml)
- Buffer
- Lepirudin (400 µg/ml)
- Bivalirudin (1 mg/ml)

The p-values are:
- p = 0.597
- p < 0.0001

Cut off
Figure 2b

OD 492nm

p = 0.227

p < 0.0001

p < 0.0001

cut off

coating of microtiter plate with

Lepirudin (10 µg/well)

Lepirudin (10 µg/well)

Lepirudin (10 µg/well)

Bivalirudin (10 µg/well)

Bivalirudin (10 µg/well)

Bivalirudin (10 µg/well)

preincubation of sera with

Buffer (400 µg/ml)

Bivalirudin (10 µg/well)

Buffer (400 µg/ml)

Bivalirudin (10 µg/well)

Bivalirudin (10 µg/well)
Antibodies against lepirudin are polyspecific and recognize epitopes on bivalirudin

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