Antibody-based strategies for the detection of Luspatercept (ACE-536) in human serum

Christian Reichel,* Günter Gmeiner and Mario Thevis

Luspatercept (ACE-536, ACVR2B-Fc), a fusion protein consisting of the extracellular domain of ActRIIB receptor and the Fc-part of human immunoglobulin G1 (IgG1), is currently under clinical development (Phase III). It stimulates the formation of red blood cells and hence may be misused by athletes for doping purposes in the future. Several antibody-based strategies for the detection of Luspatercept and other ACVR2B-Fc fusion proteins in human serum were evaluated (ELISA; IEF-, SDS-, and SAR-PAGE followed by Western blotting; immunoprecipitation). Two methods led to useful results: a commercial “soluble” ACTR-IIB ELISA, which also detected Luspatercept and other ACVR2B-Fc’s, but showed no cross-reactivity with Sotatercept/ACVR2A-Fc’s. The ELISA might be applied as fast screening tool (100 μL serum; limit of detection (LOD) ca. 15.6 ng/mL). The second method uses a polyclonal ACVR2B-antibody for immunoprecipitation followed by SAR-PAGE and Western blotting with a monoclonal detection antibody (50 μL serum; LOD ca. 1.0 ng/mL). It can be used for initial as well as for confirmatory testing. Due to the high doses (mg/kg) and long serum half-life of Luspatercept, both strategies may be useful in anti-doping control in the future. Copyright © 2017 John Wiley & Sons, Ltd.

Keywords: Luspatercept; ACVR2B-Fc; activin receptor; fusion protein; erythropoietin; SDS-PAGE; SAR-PAGE; IEF-PAGE; doping control

Introduction

In recent decades, pharmaceuticals which increase the formation of red blood cells (RBC; erythropoiesis) have been developed for treating anaemias. Recombinant erythropoietin (rEPO) was the first protein-based substance for the treatment of patients suffering from anaemia caused by chronic kidney disease (CKD) and was approved in 1989.[1,2] It stimulates the proliferation of early progenitor blood cells (burst forming unit-erythroid (BFU-E) cells and colony forming unit-erythroid (CFU-E) cells) via binding to the EPO-receptor (EPO-R).[3-6] Recently, an alternative route was discovered, which is based on removing activin receptor ligands. By trapping these ligands with soluble fusion proteins consisting of the extracellular domain of activin receptor IIα (ActRIIA) or activating receptor IIβ (ActRIIB) and the Fc-part of human IgG1, erythropoiesis can be also stimulated.[5,6] Activin receptor ligands are members of the TGF-β superfamily (eg. activins, BMPs, GDFs), which negatively regulate erythropoiesis mainly via the SMAD2/3 pathway. Upon removing the ligands, SMAD-signalling gets inhibited and thus maturation of RBC is promoted. In contrast to EPO, soluble ACVR2-traps (Sotatercept (ACE-11)/ACVR2A-Fc, Luspatercept (ACE-536)/ACVR2B-Fc) promote late stage erythropoiesis (ie, differentiation of erythroblast cells to RBC).[7-10] Luspatercept has been mainly developed for rare anaemias like β-thalassemia or myelodysplastic syndromes (MDS), which do not respond to treatment with rEPO.[9] Contrary to erythropoietin, ACVR2-traps must be applied at relatively high doses (mg/kg) and exhibit long serum half-lives (eg, 15–16 days for Luspatercept).[10] Currently, Luspatercept is in clinical phase III.[11] Doping with Luspatercept and other non-approved substances is prohibited according to the Prohibited List 2017 of the World Anti-Doping Agency (WADA; articles 50 and 52).[12] In order to detect a potential misuse by athletes, new test methods have to be developed, since Luspatercept is undetectable by the present ESA (erythropoiesis stimulating agents)-detection methods. Due to its high molecular mass (>100 kDa), blood will be the matrix of choice for anti-doping control.

Experimental

Materials

Luspatercept, consisting of the soluble part of activating receptor type-2B (N-terminal; E25-T131, modified at L79→D; Uniprot accession number Q13705) fused to the Fc-part (hinge-, CH2-, and CH3-regions) of human immunoglobulin G1 (IgG1; C-terminal) via a short linker (GGG), was expressed in Chinese Hamster Ovary cells (CHO; 335 aa) and custom synthesized by Creative Biomart (Shirley, NY, USA) and was expressed in NS0, HEK293 cells, respectively. All proteins were homodimers linked together by disulphide bridges. Information regarding their amino acid sequences can be found in Table 1.

Keywords: Luspatercept; ACVR2B-Fc; activin receptor; fusion protein; erythropoietin; SDS-PAGE; SAR-PAGE; IEF-PAGE; doping control

Table 1

<table>
<thead>
<tr>
<th>Protein</th>
<th>Description</th>
<th>Source</th>
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<tr>
<td>Luspatercept (ACE-536)</td>
<td>Fusion protein consisting of the extracellular domain of ActRIIB receptor and the Fc-part of human immunoglobulin G1 (IgG1)</td>
<td>Creative Biomart (Shirley, NY, USA)</td>
</tr>
<tr>
<td>Sotatercept (ACE-11)</td>
<td>Fusion protein consisting of the soluble part of ActRIIA receptor and the Fc-part of human immunoglobulin G1 (IgG1)</td>
<td>Creative Biomart (Shirley, NY, USA)</td>
</tr>
</tbody>
</table>

* Correspondence to: Christian Reichel, Doping Control Laboratory, AIT Seibersdorf Laboratories, A-2444 Seibersdorf, Austria. E-mail: christian.reichel@seibersdorf-laboratories.at

a Doping Control Laboratory, Seibersdorf Labor GmbH, An der Bundesstrasse 60, A-2444 Seibersdorf, Austria

b Institute of Biochemistry / Center for Preventive Doping Research, German Sport University Cologne, Am Sportpark Muenstersberg 6, 50933 Cologne, Germany
c European Monitoring Center for Emerging Doping Agents, German Sport University Cologne, Am Sportpark Muenstersberg 6, 50933 Cologne, Germany
Human recombinant erythropoietins were from Janssen-Cilag (Erypo (epoetin alpha); Vienna, Austria), Shire (Dynepe (epoetin delta); Basingstoke, Hampshire, UK), Roche (NeoRecombin (epoetin beta) and MIRCERA; Mannheim, Germany), Amgen (Aranesp (NESP); Thousand Oaks, CA USA), and ProSpec (recombinant human EPO-alpha/Fc chimera (EPO-Fc); Ness-Ziona, Israel).

Sotatercept and other ACVR2A-Fc fusion proteins used for cross-reactivity testing were either custom synthesized (Sotatercept; Creative Biomart, Shirley, NY, USA) or bought from Creative Biomart and R&D Systems (Minneapolis, MN, USA) (Table 2).

**Samples**

Human serum was bought from Sigma-Aldrich (St Louis, MO, USA) and was used for all spiking experiments.

**ACVR2B-Fc ELISA kit**

An enzyme-linked immunosorbent assay (ELISA) kit for measuring ACVR2B in human serum and plasma (human ACTR-IIB (soluble) ELISA) was purchased from Creative Diagnostics (Shirley, NY, USA). The microplate reader (Victor^3^V) was from Perkin Elmer (Waltham, MA, USA).

**Isoelectric focusing (IEF-PAGE), 2D-PAGE**

Acrylamide/bisacrylamide solution (40% T, 3% C; PlusOne ReadySol IEF), ammonium perosulfate (APS), N,N,N',N'-tetramethylethylene diamine (TEMED), urea, as well as the flat-bed electrophoresis chamber (Multiphor II), electrode strips, gel support film (GelBond PAGfilm, 124 x 258 mm), clamps (FlexiClamps) and moulds (rubber U-framed, 125 x 260 x 1.0 mm) for casting polyacrylamide gels for isoelectric focusing (IEF-PAGE) were received from GE Healthcare. Carrier ampholytes (Servalytes 2-4, 4-6, 6-8) were bought from Serva. Tween-20 solution (Surfact-108-110 A; Pierce Rockford, IL). Bovine serum albumin (BSA; ELISA grade), sucrose (electrophoresis grade), and phos-foric acid (p.a.) was from Merck (Darmstadt, Germany).

The Ettan IPGphor 3 isoelectric focusing unit for performing IEF with immobilized pH-gradient gels (IPG), IPG-strips (Immobiline DryStrips pH 3-10/7 cm, pH 4-7/11 cm), IPG buffers 3-10 and 4-7, Immobiline DryStrip Reswelling Tray, Immobiline DryStrip Cover Fluid, DeStreak Rehydration Solution as well glycine (Glycine 20x, 1 M) was from GE Healthcare. Carrier ampholytes (Servalytes 2-4, 4-6, 6-8) were from Serva. Tween-20 solution (Surfact-Amp; 10%) was from Thermo/Pierce (Rockford, IL). Bovine serum albumin (BSA; ELISA grade), sucrose (electrophoresis grade), and phos-foric acid (p.a.) was from Merck (Darmstadt, Germany).

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**SDS/PAGE**

NuPAGE BisTris precast gels (Mini: 10% T/1.5 mm/10 wells; Midi: 10% T/1.0 mm/20 wells) as well as electrophoresis chambers (Xcell SureLock Cells), empty gel cassettes, 4-morpholinopropanesulfonic acid (MOPS) running buffer (20x), lithium dodecyl sulfate (LDS) sample buffer (4x) and molecular weight marker proteins (Mark12 Unstained Protein Standard) for performing sarcosyl and sodium dodecylsulphate polyacrylamide gel electrophoreses (SAR-PAGE, SDS-PAGE) were obtained from Invitrogen (Thermo Fisher Scientific; Waltham, MA, USA). Sodium
N-lauroylsarcosinate (sarcosyl), phenol red, MOPS, glycerol, tris(hydroxymethyl)aminomethane hydrochloride (Tris-HCl), DL-dithiothreitol (DTT), ethylenediaminetetraacetic acid (EDTA, free acid), and sodium metabisulfite (Na₂S₂O₅) were from Sigma-Aldrich (St Louis, MO, USA). Tris(hydroxymethyl)aminomethane (Tris; plusOne), sodium dodecyl sulfate (SDS; plusOne) and the EPS 3500 XL power supply were bought from GE Healthcare (Uppsala, Sweden). Coomassie Brilliant Blue R-250 was obtained from Serva (Heidelberg, Germany). Hydrochloric acid (p.a.), glacial acetic acid (p.a.), and methanol (LiChrosolv, gradient grade) were from Merck (Darmstadt, Germany).

### Immunoaffinity purification

For immunoaffinity purification of Luspatercept and ACVR2B-Fc, Servalytes 2-10 (pH 2–10; FMC BioPolymer, Rockford, IL, USA) were used. The rotator (Stuart model SB3) was from Bibby Scientific Limited (Stone, UK). Ultrafree-MC centrifugal filters (0.2 μm) for microfiltration and Microcon YM-30 ultrafiltration devices (nominal molecular weight limit (NMWL) 30 kDa) were purchased from Millipore (Billerica, MA, USA).

### Western Blotting of SAR- and IEF-PAGE gels

Western blots were performed using a semi-dry blotter from BioRad (Trans-Blot SD; Hercules, CA, USA) and blotting papers from GE Healthcare (Novablot, thin "Blotting Paper 21x26cm") or BioRad (extra thick blotting paper). Blotting membranes (Immobilon-P, Durapore) were purchased from Millipore (Billerica, MA, USA). Phosphate-buffered saline (PBS) tablets were from Medicago (Uppsala, Sweden) and the low-fat dry milk from Régilait (Macon Cedex, France). Monoclonal and polyclonal antibodies directed against human activin RIIB were bought from R&D Systems (monoclonal mouse IgG1 clones #60401, #60402, and #60408, against human activin RIIB were bought from R&D Systems (Cedex, France). Monoclonal and polyclonal antibodies directed against human activin RIIB were bought from R&D Systems (monoclonal mouse IgG1 clones #60401, #60402, and #60408, polyclonal goat as well as biotinylated polyclonal goat IgGs; immunogen: Ser19-Thr134 of ACVR2B). For additional details refer to Table 3.

The biotinylated goat anti-mouse IgG (H+L) secondary antibody (ImmunoPure #31800) and the HRP-coupled goat anti-mouse IgG (H+L) cross-adsorbed secondary antibody (ImmunoPure #31432), as well as the streptavidin horseradish peroxidase (HRP) complex were obtained from Thermo/Pierce and Biospa (Milan, Italy), respectively. West Femto substrate for enhanced chemiluminescence detection was also bought from Thermo/Pierce. Western blot images were acquired using a LAS-4000 CCD-camera (Fujifilm; Tokyo, Japan) and images were analysed with GASepo software (version 2.3; Seibersdorf, Austria).  

### Methods

#### Samples

Serum was microfiltrated using Ultrafree-MC centrifugal filters (14,000g/15min) and spiked with various amounts of Luspatercept and other ACVR2B-Fc’s (NSO-, S21-derived, biotinylated). For the soluble ACTR-IR ELISA experiments, 2-fold dilution series of Luspatercept/ACVR2B-Fc’s were prepared ranging from 1000 to 25 ng/mL (determination of LOD). For ELISA cross-reactivity testing, Luspatercept and ACVR2A-Fc’s (Table 2) were spiked into serum at concentrations from 62.5 ng/mL to 4 μg/mL.

The immunoprecipitation protocol was developed using a 2-fold dilution series of Luspatercept in serum (10 to 0.5 ng/mL).

#### Measurement of Luspatercept and ACVR2B-Fc’s in serum by ELISA

The instructions of the kit were closely followed. Briefly, 100 μL of standards/blanks/spiked sera were pipetted into the ELISA wells of the kit and incubated at room temperature (RT) for 2 hours under shaking (600 rpm). After aspiration and washing (4x300 μL Washing Buffer), 100 μL of Detection Antibody working solution were added and the plate shaken again at RT/600 rpm/2 hours. After another 4 washes (300 μL each), 100 μL of Anti-Rabbit IgG-HRP Conjugate working solution were added (RT/600 rpm/1 hour). Subsequently, the wells were washed again (4x300 μL) and 100 μL of Substrate Solution were added (RT/10–20 min/dark). The reaction was stopped by addition of 100 μL Stop Solution. Finally, the absorption was measured at 450 nm using a microplate reader.

#### Isoelectric focusing in carrier ampholyte polyacrylamide gels (IEF-PAGE)

Carrier ampholytes (pH 2-6) for isoelectric focusing were prepared as described by Lasne et al.  

### Table 2. Tested ACVR2A-Fc proteins and their suppliers

<table>
<thead>
<tr>
<th>Name</th>
<th>Expression system</th>
<th>Supplier</th>
</tr>
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<tr>
<td>Sotatercept</td>
<td>CHO</td>
<td>Creative Biomart</td>
</tr>
<tr>
<td>Recombinant Human Activin RIIA Fc Chimera</td>
<td>CHO</td>
<td>R&amp;D Systems</td>
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<tr>
<td>Recombinant Human Activin RIIA Fc Chimera, 6-His-tagged</td>
<td>S21</td>
<td>R&amp;D Systems</td>
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<tr>
<td>Recombinant Human ACVR2A protein, Fc-tagged</td>
<td>HEK293</td>
<td>Creative Biomart</td>
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<tr>
<td>Recombinant Human ACVR2A, Fc-tagged, Biotinylated</td>
<td>Human cells</td>
<td>Creative Biomart</td>
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### Table 3. Evaluated antibodies for the detection of Luspatercept by immunoprecipitation and Western blotting (R&D Systems)

<table>
<thead>
<tr>
<th>Name</th>
<th>Clonality</th>
<th>Host</th>
<th>Order number</th>
</tr>
</thead>
<tbody>
<tr>
<td>Human Activin RIIB Antibody</td>
<td>Polyclonal</td>
<td>Goat IgG</td>
<td>AF339</td>
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<tr>
<td>Human Activin RIIB Biotinylated Antibody</td>
<td>Polyclonal</td>
<td>Goat IgG</td>
<td>BAF339</td>
</tr>
<tr>
<td>Human Activin RIIA/B Antibody</td>
<td>Monoclonal</td>
<td>Mouse IgG1 Clone #60401</td>
<td>MAB3391</td>
</tr>
<tr>
<td>Human Activin RIIB Antibody</td>
<td>Monoclonal</td>
<td>Mouse IgG1 Clone #60402</td>
<td>MAB3393</td>
</tr>
<tr>
<td>Human Activin RIIB Antibody</td>
<td>Monoclonal</td>
<td>Mouse IgG1 Clone #60408</td>
<td>MAB3392</td>
</tr>
</tbody>
</table>
4–6 (mixed 1:1; 4% w/v) were added to a solution containing acrylamide-bisacrylamide (5% T/3% C), 7 M urea, and sucrose (5%). After degassing and addition of TEMED and APS for polymerisation, gels with wells were cast on GelBond PAG film and polymerised overnight as described elsewhere. 

Sarayl-6–8 (2% w/v; diluted in water) and 0.5 M phosphoric acid were used as cathophores and anolytes, respectively. Methyl red (0.1% in methanol) was added to the catholyte for monitoring electrophoresis. Gels were run on a Multiphor II instrument under cooled conditions (10°C). After pre-focusing at constant voltage (250 V/30 min), different amounts of Luspatercept (800 to 3.12 pg; diluted in 0.05% BSA/PBS containing 1% Tween-20) were applied into the wells on the cathodic side of the gel and then focused for 3600–4000 Vh at constant power (25 W). The inter-electrode distance was 10 cm. After focusing, the gel was blotted onto an Immobilon-P membrane as described in the next section.

2D separation of Luspatercept (2D-PAGE)

In case of 2D-PAGE, Luspatercept was first separated by charge with immobilized pH gradient gels (IPG-strips; pH 4–7/11 cm). Luspatercept (15 μg) was dissolved in 200 μL of DeStreak solution containing 2 μL IPG-buffer (pH 4–7) and DTT (25 mM). Subsequently, IPG-strips were rehydrated in this solution at room temperature/overnight (DryStrip Resewing Tray with Immobiline DryStrip Cover Fluid for protection from drying). After rehydration, the strips were placed on the ceramic tray of the IPGphor 3 instrument and run under cooled conditions (20°C) according to a 4-step protocol provided by the supplier of the strips (slightly modified; 500 V/500 Vh/step-hold (step 1), 1000 V/1000 Vh/gradient (step 2), 6000 V/8800 Vh/gradient (step 3), 6000 V/8000 Vh/step-hold (step 4)). In total, proteins were focused for 18300 Vh. After IEF, the strips were transferred to SDS-PAGE gels for further separation by mass. Hand-cast Laemmli Tris-HCl Midi-gels (10% T, 1 mm, without stacking gel) were used for SDS-PAGE separation. For these gels, 10 mL of acrylamide/bisacrylamide solution (PlusOne ReadySol IEF) were mixed with 10 μL of 1 M Tris-HCl buffer (pH 8.8) and 19.1 mL water, degassed for 10 minutes and supplemented with 400 μL of 10% SDS-solution. Then 16 μL of TEMED and 400 μL of 10% APS-solution were added to initiate polymerization. The mixture was filled into an empty gel cassette, overlayed with water, and allowed to polymerize for at least 2 hours at room temperature. Immediately before SDS-PAGE, IPG-strips were first equilibrated 2×10 min in reducing buffer (60 mM Tris-HCl, pH 6.8) containing urea (6M), SDS (2%), glycerol (10%), and DTT (65 mM) and subsequently 2×10 min in an alkylating buffer (60 mM Tris-HCl, pH 6.8 containing 135 mM IAA). Strips were then put on top of the manually cast Tris-HCl gels and fixed with sealing solution (125 mM Tris-HCl buffer pH 6.8 containing 1.5% low melting point agarose; 55°C). Electrorophoresis was performed at constant voltage (150 V) for 2 hours. The running buffer contained 25 mM Tris, 192 mM glycine, and 0.1% SDS. After electrophoresis, gels were stained with Coomassie R-250.

Sarcosyl (SAR)- and SDS-PAGE for 1D separation

SAR- and SDS-PAGE were described as elsewhere with minor modifications. Briefly, SAR-sample buffer (SAR-SB; 4-fold concentrated; 424 mM Tris–hydrochloride, 564 mM Tris, 8% SAR, 40% glycerol, 2.04 mM EDTA), SAR-running buffer (SAR-RB; 50 mM MOPS, 50 mM Tris, 0.1% sarcosyl, 1 mM EDTA), sodium metabisulfite solution (antioxidant; 380 mg Na₂S₂O₅ dissolved in 1 mL water), and DTT-solution (reducing agent; 4M in water) were prepared before electrophoresis. 10% T BisTris Mini (1.5 mm, 10 wells) and Midi (1.0 mm, 20 wells) precast gels were used for all experiments. For the catholyte, 200 μL (Mini gels)/400 μL (Midi gels) of SAR-RB were supplemented with 500 μL/1000 μL of antioxidant and filled into the inner buffer chamber of the electrophoresis cell. Then the gel wells were washed with this buffer. For antibody evaluation on Western blots (LOD), 2-fold dilution series of Luspatercept and ACVR2B-Fc standards in SAR-SB (1x) were prepared ranging from 800 to 3.125 pg (pretesting) and 800 to 0.78 pg (LOD-determination; absolute amount on gel). Immunooactivity-purified samples were obtained as described later. For gels stained with Coomassie R-250 (estimation of apparent molecular mass), 1 μg of each standard was applied on gel. All standards and samples were mixed with 3.75 μL SAR-SB (4x), 1.5 μL reducing agent, filled up with water to 15 μL, and then heated at 95°C/5 minutes/650 rpm. After cooling down, they were loaded in the gel wells and anolytes (SAR-RB without antioxidant) was filled into the outer chamber. The gels were run at constant voltage (200V/1h20 min/Midi-gels, 200V/1 h/Mini-gels; cooled) and then either stained with Coomassie R-250 or blotted. For SDS-PAGE gels, the same procedure was used except that SAR-SB and SAR-RB were replaced by LDS-SB and MOPS-RB as supplied by the manufacturer, respectively.

Coomassie R-250 stain of SDS/SAR-PAGE gels

Gels were first fixed for 15 minutes in a solution containing 50% methanol/10% acetic acid and then stained overnight in 0.025% Coomassie R-250/40% methanol/10% acetic acid. For destaining, a solution of 20% methanol/10% acetic acid was used.

Semi-dry Western blotting

SAR-PAGE gels were semi-dry blotted for 60 minutes at constant current (1.56 mA/cm²) using a modified Bjerrum blotting buffer (48 mM Tris/39 mM glycine/0.0375% SAR/20% methanol) and Immobilon-P membranes, which were activated before usage for 30 seconds in methanol followed by water washing and equilibration in blotting buffer. Before blotting, the gels were equilibrated for 5 minutes in blotting buffer. One layer of extra thick blotting paper was used on each side of the sandwich consisting of blotting paper/membrane/gel/blotting paper. After the blot, membranes were briefly washed in PBS, incubated in DTT-solution (10mM in PBS) at 37°C for 60 minutes, washed again in PBS, and then blocked with low-fat milk (5% LFM/PBS; 60 minutes). Following another PBS-wash, they were incubated in primary antibodies at the concentration recommended by the supplier (1 μg/mL in 1% LFM/PBS for clones #60401, #60402, and #60408, and 0.1 μg/mL for the biotinylated polyclonal antibody; see-saw shaker; 15 rpm/coldroom/overnight), washed with 0.5% LFM/PBS (3 x 10 min), then rinsed briefly with PBS, incubated in biotinylated secondary antibody (reconstituted according to the instructions of the supplier and diluted 1:2000 in 1% LFM/PBS; 15 rpm/RT/60 minutes), and washed again with 0.5% LFM/PBS (3 x 10 min). After another PBS-rinse the membranes were incubated in streptavidin-HRP-complex (1:2000, 1% LFM/PBS, 15 rpm/RT/60 minutes), washed in PBS (3 x 10 minutes), and finally developed with West femto chemiluminescence substrate. In case of the biotinylated polyclonal anti-ACVR2B antibody, membranes were directly incubated in streptavidin-HRP solution after 0.5% LFM/PBS washing (3 x 10 minutes). In addition to these single-blot, double-
blotting was applied to investigate non-specific interactions of the biotinylated secondary antibody. The method was performed as described by Lasne et al.[25] Briefly, after incubation with primary antibody and washing (0.5% LFM/PBS, 3x10 minutes), the bound antibodies were transferred under acidic conditions (0.7% acetic acid, 0.8 mA/cm², 10 minutes) to a second Immobilon-P membrane. Subsequently, the membrane was blocked and incubated in biotinylated secondary antibody as well as streptavidin-HRP as described earlier. Images were acquired in automatic and manual modes of the LAS-4000 camera. For IEF-PAGE gels the same protocol was applied, except that instead of Bjerrum buffer, Towbin buffer (25mM Tris, 192mM glycine, without methanol) was used for blotting after the gel was equilibrated for 2 minutes in buffer and GelBond PAG film removed (3 layers of thick blotting paper on each side; Durapore membrane between gel and Immobilon-P membrane; 1mA/cm², 30 minutes).[25]

**Immunofinity purification of Luspatercept and ACVR2B-Fc’s from serum**

Pierce Direct IP Kit was used for immunoaffinity extraction of Luspatercept and other types of ACVR2B-Fc’s from serum samples. The instructions of the kit were followed closely for antibody coupling to the resin, immunoprecipitation, and elution of the bound fraction.[26] Briefly, all reagents were brought to room temperature (RT) and then 20 μL of resin slurry per sample were transferred into a spin column provided with the kit, centrifuged (1000g/1 min), and washed twice with 200 μL of coupling buffer (0.01M sodium phosphate, 0.15M NaCl, pH7.2). For antibody immobilization, 10 μg of antibody (polyclonal goat IgG ActRIIB or monoclonal mouse clone #60408 antibody) were mixed with 10 μL of coupling buffer (20x), filled up with MQ-water to 200 μL, and mixed with the washed beads inside the spin column. Then 3 μL of sodium cyanoborohydride solution (NaBH₃CN, 5M) were added and the mixture was incubated for 90 minutes at room temperature under shaking (Thermomixer, 600 rpm). Afterwards the solution was removed by centrifugation and by washing the beads twice with 200 μL of coupling buffer (1x). Subsequently, the beads were washed once with 200 μL of AminoLink Quenching buffer (1M Tris-HCl), then another 200 μL of quenching buffer were added plus 3 μL of NaBH₃CN solution (room temperature/15 min/400 rpm Thermomixer). After another centrifugation step the beads were washed again 2 times with 200 μL coupling buffer followed by 6 washes (150 μL each) with Wash Solution (1M NaCl). For immunoprecipitation, the beads were finally washed with cold IP Lysis/Wash Buffer (0.025 M Tris, 0.15 M NaCl, 0.001 M EDTA, 1% NP-40, 5% glycerol, pH 7.4; 2x 200 μL) and then 50 μL serum sample plus 450 μL IP Lysis/Wash Buffer were added. The mixture was incubated overnight under rotation (Stuart rotator; 10 rpm) in the coldroom. After centrifugation, the beads were washed 4 times with 200 μL IP Lysis/Wash Buffer and one time with 100 μL of Conditioning Buffer (1x). For elution, a total of 100 μL of Elution Buffer (pH2.8; 25+75 μL) containing 0.1% Tween-20 were added and the eluate was collected after centrifugation (1000g/1min). The eluate was immediately neutralized by addition of 5 μL Tris-HCl buffer (3.75 M, pH9.2) and was further concentrated down to < 10 μL by ultrafiltration with Microcon YM-30 devices (14000g/ca 6 minutes, 1000g/1min for retentate recovery). Subsequently, 3.75 μL SAR-sample buffer (4x) and 1.5 μL DTT solution (1M) were added and the retentate was filled-up to 15 μL with MQ water. Retentates were stored at -20°C until electrophoresis.

**Results and discussion**

Current testing strategies for detecting ESAs in doping control are mainly based on electrophoretic techniques (IEF-, SDS/SAR-PAGE) combined with immunological methods (immunoaffinity purification, Western blotting). Since anti-EPO antibodies do not bind to Luspatercept and other ACVR2B-Fc’s, a modified strategy had to be developed. Antibody-based techniques such as ELISA, immunoprecipitation, and Western blotting were evaluated regarding their capability to detect Luspatercept/ACVR2B-Fc’s in serum at physiologically relevant concentrations.

**Performance characteristics of Luspatercept on SDS- and SAR-PAGE**

For evaluating the performance characteristics of Luspatercept and HEK293-expressed ACVR2B-Fc after molecular mass based electrophoretic separation, SDS- and SAR-PAGE analyses were performed under identical conditions (10% T BisTris gels, 1.5 mm, 10 wells; 200V constant; 60 minutes). Band position and band shape were compared with epoetins (Erypo, NeoRecormon, NESP, Dynepo, Mircera, EPO-Fc) after Coomassie R-250 stain. The main aim was to assess if ACVR2B-Fc’s can be differentiated by methods, which are currently used for initial and confirmatory testing (SAR-PAGE) or just for confirmation (SDS-PAGE) in doping control (WADA TD2014EPO).[27] As shown in Figure 1, both ACVR2B-Fc’s – regardless whether expressed in CHO- or HEK-cells – can be distinguished by their apparent molecular masses from other ESAs. Under reducing conditions, ACVR2B-Fc’s migrate as broad bands between 50 and 60 kDa (SDS-PAGE). They are well separated from epoetins. Due to N-glycosylation (N18, N41, N185), the apparent molecular mass of Luspatercept under reducing conditions is higher than the theoretical mass of its homomers (ca 38–39 kDa). Compared to SDS-PAGE, the band shape is considerably narrower on SAR-PAGE. Figure 1 also demonstrates the enhanced performance characteristics of SAR-PAGE for CERA and EPO-Fc.[22,28] Based on these data, SAR-PAGE and SDS-PAGE can be used for the differentiation of Luspatercept and ACVR2B-Fc’s from EPO-based ESAs by molecular mass, ie, with methods already in the scope of TD2014EPO.

**Antibody evaluation for Western blotting**

In total, 4 antibodies were tested – 3 monoclonals (clones #60401, #60404, and #60408) and one polyclonal. All antibodies were directed against the extracellular domain of ACVR2B (aa19–134), ie, the functional region of Luspatercept and other ACVR2B-Fc fusion proteins. SAR-PAGE with Western blotting was used for the evaluation. For pretesting a concentration range of 800 ng to 3.125 pg was investigated. The results for Luspatercept are shown in Figure 2. The highest sensitivity was obtained for clone #60408 followed by the biotinylated polyclonal goat IgG. Both antibodies detected 3.125 pg Luspatercept on gel (60 seconds exposure time/ CCD-camera in standard sensitivity mode). Under the same conditions, clone #60402 detected only 200 pg of the fusion protein and clone #60401 no Luspatercept at all. However, the 2 antibodies performed better with the NS0-expressed ACVR2B-Fc (ca 25 pg), but clone #60401 required 120 seconds exposure time in high sensitivity mode (data not shown). This might be explained by the fact that the same NS0-expressed protein was used as immunogen for generating these antibodies but not Luspatercept.

Due to the fact that clone #60408 showed the highest sensitivity, it was used for all subsequent Western blot experiments including
the LOD-determination of Luspatercept. The lowest detectable amount was 0.78 pg on gel (Figure 3).

IEF-PAGE of Luspatercept with carrier ampholytes

The main purpose of this test was to investigate if Luspatercept can be separated into isoforms under conditions generally used for the doping-related detection and differentiation of various erythropoietins (pH 2–6 carrier ampholyte-based IEF-gels containing 7M urea; ca 10 cm inter-electrode distance). A dilution series (800 pg to 3.12 pg) of Luspatercept in 0.05% BSA/Tris-HCl (50mM, pH 7.4) was applied on the gel. Similar to epoetins, 1%Tween-20 was added for additional solubilisation. Regardless of whether heat-denaturation was used or not, Luspatercept could not be separated into discrete isoforms. Only a smear-like array of potential isoforms was obtained starting directly at the application point (pH 6) and ranging approximately to one third of the gel (pH 4). Even amounts in the very low picogram-range could not be successfully separated into isoforms (Supporting Information). Most likely, the resolution of the carrier ampholyte gels was insufficient to resolve the many isoforms present. Hence, IPG-IEF was also tested for further clarification (Section 2D-PAGE).
IPG-IEF and 2D-PAGE of ACVR2B-Fc

Isoelectric focusing in immobilized pH-gradient gels (IPG-IEF) poses several advantages over CA IEF-PAGE, namely the application higher voltage gradients (up to ca 500V/cm) and very high resolution within 0.01pH units depending on the length of the IPG-strips.[20] Disadvantages are that focusing may take much longer and that the strips cannot be electroblotted like carrier ampholyte-based or SDS/SAR-PAGE gels due to the non-removability of the plastic backing. Consequently, the strips have to be either “press-blotted”[28] or transferred to SDS-PAGE for transferring the isoforms out of the IPG strip into the SDS-PAGE gel (classical 2D-PAGE). Also staining of the strips is more complicated than staining of SDS-PAGE gels.

For pre-testing, pH 3-10 IPG-strips (7 cm) were used as described elsewhere.[29] After 2D-separation and Coomassie staining, they revealed, that Luspatercept focuses between ca pH 4 and pH 6 as already expected from the CA-IEF-PAGE results. However, the resolution was still too low to obtain good separation of the more acidic isoforms. Hence, it was decided to use longer IPG-strips (11 cm) with a narrower pH-range (pH 4–7; 15 μg protein on gel). Under these conditions, acceptable focusing results were achieved and about 23 isoforms were observed (Figure 4). Luspatercept contains 3 N-glycosylation sites on each monomer (N18 and N41 of the ACVR2B-chain, N185 of Fc-CH2).[13] Most likely, these post-
translational modifications contribute to the charge heterogeneity of its isoforms. However, due to the time consumed, 2D-PAGE would not be a suitable method for large scale, routine doping control testing.

**Detection of Luspatercept in serum by sACVR2B-ELISA**

Since ACVR2B is a transmembrane protein and Luspatercept contains only its extracellular (“soluble”) domain, a commercial ELISA for the quantification of soluble ACVR2B was evaluated regarding its capability to detect Luspatercept and other ACVR2B-Fc’s in human serum. The kit uses a monoclonal capture and polyclonal detection anti-ACVR2B antibody as well as an HRP-coupled secondary antibody for colorimetric readout with 3,3',5,5'-Tetramethylbenzidine (TMB)-substrate (450 nm). Serum was spiked with Luspatercept ranging from 0 ng/mL to 1 μg/mL. As shown in Figure 5, the ELISA could detect Luspatercept down to 15.6 ng/mL. Similar results were obtained for NS0- and Sf21-expressed ACVR2B-Fc proteins. No cross-reactivity with Sotatercept and other ACVR2A-Fc’s was observed up to 4 μg/mL. Hence, the ELISA can be also used for differentiating between samples containing Luspatercept and Sotatercept.

**Figure 6.** Immunoaffinity purification of Luspatercept from serum followed by SAR-PAGE and Western blotting for detection (clone #60408 antibody). [Colour figure can be viewed at wileyonlinelibrary.com]

**Figure 7.** Detection of Luspatercept in serum by immunoaffinity purification, SAR-PAGE and Western blotting with clone #60408 primary antibody. (A) Single blotting using a biotinylated secondary antibody (Thermo/Pierce #31800) plus streptavidin-HRP complex. (B) Single blotting using a HRP-labelled cross-adsorbed secondary antibody (Thermo/Pierce #31432). (C) Double blotting using the same combination of antibodies as on (A). Only (A) allowed the detection of Luspatercept down to 1.0 ng/mL in just 50 μL of serum. Images were lane-specifically contrast optimized. Hence bands with lower intensity appear enhanced (including background). Marked in red is the non-specific band caused by the biotinylated secondary antibody. [Colour figure can be viewed at wileyonlinelibrary.com]
**Immunoaffinity purification of Luspatercept followed by Western blot detection**

Pierce Direct IP Kit was used for immunoaffinity enrichment of Luspatercept and other ACVR2B-Fc’s from serum samples. The kit was chosen, because it was already successfully applied for the purification of Peginesatide (another ESA) from serum and plasma and because other strategies were unsuccessful due to highly non-specific binding (eg, biotinylated polyclonal goat IgG immobilized on streptavidin beads) or the necessity of additional pre-purification steps (eg, HSA-depletion with immunoaffinity beads or IgG-Fc specific enrichment with Protein A/G beads, both followed by ELISA immunoaffinity purification; data not shown). One monoclonal antibody (clone # 60408) and the non-biotinylated polyclonal goat IgG immobilized on streptavidin beads were tested with the Direct IP-kit in combination with SAR-PAGE and Western blotting. The polyclonal antibody performed better and hence was selected for all subsequent experiments. The decision was also supported by the fact that clone # 60408 was slightly more sensitive on Western blots than the biotinylated version of the polyclonal antibody. Figure 6 summarizes the steps of the final method.

For LOD determination a 2-fold dilution series of Luspatercept in serum was used (10 to 0.5 ng/mL). Only 50 μL of serum were used for immunoprecipitation. The developed method allowed the detection of Luspatercept down to 1.0 ng/mL, which was clearly distinguishable from blank serum and serum containing 0.5 ng/mL. However, some samples showed a faint band above the Luspatercept band (Figure 7A). As single blots were used, the band was most likely caused by the biotinylated secondary antibody. It was no longer detectable on single blots using the HRP-conjugated cross-adsorbed secondary antibody (Figure 7B) and on double blots as applied in doping control for ESA-detection in serum (Figure 7C). Since the sensitivity of the latter 2 methods was lower than for the single blot with the biotinylated secondary antibody/streptavidin-HRP system and no interference with the Luspatercept band occurred, the application of single blotting with this detection system is justified (Figure 7).

**Conclusion**

Several immunological strategies were tested in order to develop a detection method for Luspatercept and other ACVR2B-Fc fusion proteins in serum samples (ELISA, IEF-PAGE, SDS/SAR-PAGE plus Western blotting). Since no sera of human or animal administration studies were accessible, commercial serum was spiked with Luspatercept and used for method development. With a commercial ELISA directed against “soluble” ACVR2B (Creative Diagnostics), Luspatercept could be determined down to ca 15.6 ng/mL. Of the IEF-PAGE methods, only IPG-IEF (pH 4–7; performed as 2D-PAGE) allowed the separation of ACVR2B-Fc’s in isofoms due to higher voltage gradients, which are not compliant with carrier ampholyte-based IEF-gels. Both, SDS- and SAR-PAGE could differentiate Luspatercept from EPO-based ESAs (epoetins alpha/beta/delta, darbepoetin alfa, Pegylated epoetin beta, EPO-Fc) by apparent molecular mass. Since sharper bands were obtained with SAR-PAGE, it was used for developing a Western blot-based detection method. After immunoaffinity purification, Luspatercept could be detected in 50 μL serum down to 1.0 ng/mL. Considering the long serum half-life (15–16 days) and high doses required for stimulating erythropoiesis (mg/kg), both the ELISA- and SAR-PAGE methods will be able to detect Luspatercept misuse over a period of several weeks. Since the ELISA-method is less time- and material-consuming it could be used as fast screening tool. However, the SAR-PAGE method should be the preferred method due to its lower LOD and the additional ability to confirm the molecular mass of the antibody-bound protein.

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**References**


