Development of auto-antibodies towards β2-glycoprotein I in the antiphospholipid syndrome

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CHAPTER 6

Detection of lupus anticoagulant in the presence of rivaroxaban by taipan snake venom time

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According to the consensus classification criteria, an individual is diagnosed with the antiphospholipid syndrome (APS) when the following conditions are met: the persistent presence of circulating antiphospholipid antibodies and a history of thrombosis or pregnancy morbidity\(^1\). The dominant antigenic target recognized by the APS auto-antibodies is \(\beta_\text{2}-\text{Glycoprotein I (}\beta_\text{2GPI})\).\(^2\) The clinical manifestations correlate best with the prolongation of phospholipid-dependent clotting assays; the lupus anticoagulant (LAC)\(^3\). To prevent thrombotic complications, patients diagnosed with APS are maintained on anticoagulant treatment. This treatment itself prolongs clotting assays and therefore interferes with detection of the LAC. There is no consensus on the treatment of patients with APS\(^4,5\) and there is room for alternative treatment. One of the recently developed anticoagulants is rivaroxaban, a direct factor Xa inhibitor\(^6\). Rivaroxaban has been developed for both prophylaxis and treatment of thrombosis. Studies are ongoing to determine the efficacy of rivaroxaban in patients diagnosed with APS\(^7\). Here we studied the interference of rivaroxaban in different assays developed for the detection of LAC.

All plasmas were collected in 0.109 M citrate. Normal pooled plasma (NPP) was obtained from more than 200 healthy individuals. Plasmas of thirteen SLE patients were used, none of the patients received anticoagulants at the time of blood withdrawal. Six SLE patients were positive for antiphospholipid antibodies and had a history of thrombotic complications, the 7 other patients were negative for antiphospholipid antibodies and had no history of thrombosis. The study was approved by the local ethics committee and written informed consent was obtained from all healthy individuals and patients in accordance to the declaration of Helsinki. Monoclonal antibodies were added to NPP to create artificial LAC positive plasmas: one human anti-\(\beta_\text{2GPI}\) IgG against domain I (ab1, 250 µg/mL) and two mouse anti-human \(\beta_\text{2GPI}\) IgG; one directed towards domain I (ab2, 100 µg/mL), and one directed to domain IV of \(\beta_\text{2GPI}\) (ab3, 250 µg/mL). The stock of rivaroxaban was dissolved in DMSO (Dimethylsulfoxide), this was further diluted in TBS buffer (20 mM Tris, 150 mM NaCl, pH 7.4). Before use the solution was heated to 37°C to completely dissolve the rivaroxaban. Control plasmas received the same amount of DMSO. 250 ng/mL rivaroxaban was added to NPP a concentration in the middle of the therapeutic range\(^6,8\) and clotting times were recorded with a KC-10A micro coagulometer (Amelung, Lemgo, Germany). To determine the presence of LAC, three different assays were used consisting of a screen and confirmation assay. aPTT: screen PTT-LA (Diagnostica Stago, Arnières sur Seine, France) and confirm Actin FS (Siemens Healthcare). dRVVT: screen LA-1 (Siemens Healthcare Diagnostics, Marburg, Germany) and confirm LA-2 (Siemens Healthcare Diagnostics). For the Snake venom assay: screen Taipan snake venom time\(^9\) (Sigma Aldrich, St. Louis, MO) and confirm Ecarin venom time (Sigma Aldrich). For the Taipan snake venom time assay, after incubation of rivaroxaban and antibodies, 25 µL of Bell and Alton platelet substitute (cat no BAPS040, Diagen,
Thame, UK reconstituted in 10 mL of H₂O) was added and incubated for 2 minutes before activating prothrombin with 25 µL of 5 µg/mL Taipan snake venom dissolved in 25 mM CaCl₂. Ecarin time was initiated with 25 µL of 5 units/mL Ecarin venom in

**TABLE 1.** The LAC ratio was determined for normal pooled plasma (NPP), NPP spiked with anti-β₂GPI antibodies (ab1 = human monoclonal antibody against domain I; ab2 = mouse monoclonal antibody directed against domain I; ab3 = mouse monoclonal antibody against domain IV of β₂GPI) and 13 well characterized patients. The LAC ratio was determined in the absence (-) or presence (+) of rivaroxaban (250 ng/mL). The patients were selected on the presence or absence of LAC in a diagnostic setting with aPTT and dRVVT-based assays. Conditions for which the diagnosis would change due to the presence of rivaroxaban are indicated in bold. Anti-CL: anti-cardiolipin antibodies, anti-FII: anti-prothrombin antibodies. NA = not measured. A normalized LAC ratio for the dRVVT and aPTT above 1.15 was considered positive and for Taipan snake venom time/Ecarin venom time the ratio was 1.17. Cut-offs were based on the 99th percentile of a healthy population.
2.5 mM CaCl₂. The LAC ratio was calculated by dividing the normalized ratio (result test sample/result NPP) for the LAC screen test by the normalized ratio of the LAC confirm test. The addition of rivaroxaban to NPP prolonged all conventional assays (PTT-LA, Actin FS, LA-1 and LA-2). The aPTT screen and confirm assays had comparable prolongations, resulting in a normalized LAC ratio that was hardly influenced. However, for the dRVVT screen assay a stronger prolongation of the coagulation time was observed compared to the dRVVT confirm assay. This lead to an increased normalized LAC ratio, as also has been published before. Rivaroxaban did not influence either the Taipan venom time or the Ecarin time and therefore the normalized LAC ratio for the snake venom times remained stable (Table 1).

When NPP was spiked with anti-β₂GPI antibodies, all three assays to detect the presence of LAC became positive. Addition of rivaroxaban increased the LAC ratio when determined with a dRVVT. When LAC was determined with an aPTT, the ratio increased in one sample, decreased in one other, and in one it became negative. The LAC ratio of the Taipan/Ecarin clotting times in the presence of anti-β₂GPI antibodies did not change after addition of rivaroxaban.

Thirteen patients suffering from systemic lupus erythematoses (SLE) were selected. For 6 patients with a positive LAC ratio (patients A-F), the LAC ratio was not influenced when measured with the Taipan/Ecarin venom assays, but slightly increased when the LAC ratio was measured with the aPTT, and strongly influenced when the LAC ratio was measured with the dRVVT in the presence of rivaroxaban. Addition of rivaroxaban to the LAC positive plasmas did not influence the outcome of LAC determination. When rivaroxaban was added to the plasma of the 7 SLE patients (G-M) negative for LAC, different results were observed. When the LAC ratio was measured with an aPTT in the presence of rivaroxaban, 3 of the 7 patients became positive for LAC. No influence of rivaroxaban on the Taipan/Ecarin time was observed.

Here we show that the presence of rivaroxaban in plasma samples at pharmacological concentrations can change the results of LAC determinations as measured with the officially recommended assays for the detection of LAC: the aPTT and the dRVVT. The effect of rivaroxaban on the aPTT is subtle. When plasma was used from SLE patients know to be negative for LAC, approximately 40% of these plasmas became (weakly) positive after addition of rivaroxaban. An aPTT clotting time will in general only increase when the levels of one of the clotting factors that determine the aPTT decreases below ~50%. However, when a weak inhibitor is combined with slightly decreased levels of clotting factors, individually not strong enough to influence the aPTT, the combined effect could prolong the aPTT enough to become positive in LAC.
testing.
Taipan and Ecarin are two snake venoms that contain a direct activator of prothrombin. The ratio between the Taipan and Ecarin clotting times is useful to determine the presence of LAC\(^9\). Our experiments show that the Taipan/Ecarin ratio is a sensitive assay to measure the presence of LAC and that this ratio is not affected by the presence of rivaroxaban.
REFERENCES


