Prospective testing for drug-dependent antibodies reduces the incidence of thrombocytopenia observed with the small molecule glycoprotein IIb/IIIa antagonist Roxifiban: Implications for the etiology of thrombocytopenia


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List of abbreviations: CAD, coronary artery disease; DDAB, drug-dependent antibodies; ELISA, enzyme-linked immunosorbent assay; GP IIb/IIIa, glycoprotein IIb/IIIa; PAD, peripheral artery disease; PPP, platelet poor plasma.
Summary

Thrombocytopenia is a relatively common side effect observed during glycoprotein (GP) IIb/IIIa antagonist therapy. With the oral antagonist Roxifiban, we observed thrombocytopenia, defined as 50% reduction of platelets over pre-dose values or below 90,000 per microliter, with a frequency of 2 percent (8/386). Thrombocytopenia occurred either early (days 2-4) or delayed (days 11-16). No additional cases were observed with up to 6 month of treatment. Retrospective analysis provided evidence for drug-dependent antibodies (DDABs) to GP IIb/IIIa in 5 out of 6 subjects, suggestive of an immune etiology of thrombocytopenia. The hypothesis that excluding patients based on positive DDAB reaction would reduce the frequency of thrombocytopenia was tested. Patients were screened for DDABs during the study qualification period, and overall, 3.9% of the patients were excluded based on pre-existing DDAB concentrations above a statistically defined medical decision limit. Additional 2.6% were excluded based on therapy-related antibody production during the first 2 weeks. With antibody testing, 0.2 percent (2/1044) patients developed immune-mediated thrombocytopenia. One case developed a rapidly increasing antibody concentration and presented with thrombocytopenia despite discontinuation of Roxifiban therapy. The second case was related to a false negative test result. The frequency of thrombocytopenia was statistically significantly reduced from 2% to 0.2% (p=0.0007) comparing non-screened and screened patients. Testing for DDABs can reduce the frequency of thrombocytopenia in patients treated with Roxifiban and by analogy other GP IIb/IIIa antagonists. Thus, DDAB testing may be employed to increase the safety of GP IIb/IIIa antagonists.
Introduction

Thrombocytopenia has been observed with all currently approved glycoprotein IIb/IIIa antagonists (intravenous formulations) and a number of developmental candidates. Thrombocytopenic patients experience more bleeding and ischemic events \(^{(1,2)}\). In the case of approved intravenous agents, this side effect has been observed with a frequency of up to 5.6% \(^{(1-9)}\). In addition, a number of oral GP IIb/IIIa antagonists have undergone clinical testing and treatment for example with RPR 109891 was associated with a thrombocytopenia frequency of up to 13% \(^{(10)}\). Lower frequencies have been observed for the other oral agents in clinical development. For sibrafiban, the thrombocytopenia frequency was not different compared to placebo \(^{(11)}\), whereas in the case of orbofiban, increased frequencies were observed in the treatment arms \(^{(12)}\). We recently reported 2 patients with drug-induced thrombocytopenia associated with Roxifiban treatment \(^{(13)}\). The sample size was too small to obtain accurate estimates as to the frequency of thrombocytopenia or to generalize as to the prevailing pathomechanism in the patient population treated with Roxifiban.

Some general risk factors for thrombocytopenia induced by GP IIb/IIIa antagonist therapy have been identified, including advanced age, lower body weight, and lower baseline platelet count \(^{(1,2)}\). Retrospective studies in humans and preclinical animal models implicated drug-dependent antibodies to glycoprotein IIb/IIIa in the etiology of thrombocytopenia. For example, a chimpanzee presented with acute thrombocytopenia upon treatment with GP IIb/IIIa antagonists \(^{(14)}\). Platelet FACS analysis provided evidence for the presence of GP IIb/IIIa antagonist-dependent anti platelet antibodies to GP IIb/IIIa antagonist that elicited thrombocytopenia, but not to antagonists that were
well tolerated in the same chimpanzee (\textsuperscript{14}). Support for the immune nature of thrombocytopenia in humans is derived from two patients treated with Roxifiban, an orally bioavailable GP IIb/IIIa antagonist. Both patients underwent conversion to a highly positive drug-dependent antibody status temporally associated with thrombocytopenia (\textsuperscript{13}). Previous studies are consistent with the hypothesis that the antibodies bind to platelet in a Roxifiban-dependent manner via their ability to recognize Roxifiban-induced conformational changes in GP IIb/IIIa (\textsuperscript{13}).

Here, we report that thrombocytopenia is a relative common side effect during treatment with Roxifiban and occurs with a frequency of about 2\%. Thrombocytopenia was either observed early (days 2 to 4) or delayed (days 11 to 16). No additional cases occurred beyond this susceptible time period. The majority of the cases presented with drug-dependent antibodies to GP IIb/IIIa. Thus, we tested the hypothesis that prospective testing for pre-existing or increasing DDAB concentrations would reduce the incidence of thrombocytopenia in patients treated with Roxifiban. Patients were tested for the presence of pre-existing DDABs prior to enrollment and during the first two weeks of therapy. We provide evidence that controlling patient enrollment based on DDAB status significantly reduces the incidence of GP IIb/IIIa antagonist induced thrombocytopenia, further supporting the immune etiology of GP IIb/IIIa antagonist-induced thrombocytopenia.

**Materials and Methods**

**Study populations.** Specimens for DDAB testing were collected during phase II clinical trials of Roxifiban. DMP 754 - 010 (Parts C and D) and DMP 754-017 were randomized,
double-blind, multi-center, multi-dose studies of the GPIIb/IIIa antagonist Roxifiban. Inclusion criteria included documented coronary artery disease (CAD; myocardial infarction, PTCA, angiography, unstable angina, CABG) within 3 months or documented CAD plus an additional risk factor (60+ years, diabetes, smoking). Estimates as to the frequency of thrombocytopenia in the absence of DDAB testing were derived from DMP 754-010 (Parts A and B; CAD population), DMP 754-008 (CAD population), and DMP 754-302. The latter study was conducted using patients with advanced peripheral artery disease (ankle-to-brachial index 0.6 or toe-to-brachial index 0.4). The retrospective testing for DDABs in study DMP 754-010, parts A and B has been reported (13).

Thrombocytopenia is defined as reduction of the platelet number to below 90,000 per microliter or below 50 percent compared to pre-dose values. In general, clinical samples were obtained at least 12 hours after the last treatment with Roxifiban. Roxifiban has a high affinity for resting platelets and the free concentration of Roxifiban in plasma is less than 1% of total drug, avoiding interference with the differential ELISA (see below).

Quantification of free DDABs in plasma. Blood from patients in DMP754-010 studies was collected into 1:10 volume of 3.2% sodium citrate. Platelet poor plasma (PPP) was prepared by centrifugation at 1,500g for 15 minutes and stored at <-15°C or <-60°C. Plasma was analyzed by a differential ELISA using GP IIb/IIIa passively absorbed to microtiter wells (13). Briefly, plasma diluted 1/20 was analyzed in the absence or presence of Roxifiban and bound IgG was detected with HRP-labeled polyclonal goat anti-human IgG (Kirkegaard & Perry; Gaithersburg,MD) antibodies followed by 3,3',5,5'-tetramethylbenzidine (Pierce). The detecting antibody reacts with human IgG 1 to 4, but
not with human IgM (not shown). Plates were washed between the incubation steps with an automated multi-reagent plate washer (Model AM60; Dynatech Laboratories Inc.).

**Quantification of platelet-bound and free DDABs.** DDABs to Roxifiban bind to conformationally sensitive epitopes in GP IIb/IIIa \(^{13}\). EDTA treatment of platelets at \(37^\circ\)C results in the dissociation of the GP IIb/IIIa complex on intact platelets \(^{15-18}\), leading to the release of bound antibodies that recognize complex-specific determinants induced by Roxifiban \(^{13}\). After removal of platelets by centrifugation, the resulting plasma is expected to contain free and previously platelet bound conformationally sensitive DDABs. To test whether quantification of the platelet-bound and free pool of DDABs increase the sensitivity of the DDAB ELISA, blood from patients enrolled in DMP754-017 was collected into vacutainers containing 15 % EDTA, heat treated at \(37^\circ\)C for 1 hour with end-over-end rocking, and PPP was prepared as above. A parallel sample was also collected in sodium citrate and archived.

EDTA treatment also dissociates Roxifiban from the platelet surface, precluding the direct analysis of the plasma by the differential ELISA (antibody binding in the presence and absence of Roxifiban). A Roxifiban removal step with C18 (Orochem), a hydrophobic matrix with high affinity for Roxifiban, was included. Briefly, 50 mg of the hydrophobic matrix was activated with 100 % methanol and then equilibrated with buffer. EDTA PPP (500 ul containing 100 nM DuP 714 [final concentration], an in-house thrombin inhibitor, and 0.32 % sodium citrate) was incubated with the matrix for 15 minutes. PPP was recovered by centrifugation at 2,500 g for 5 minutes. Control experiments (not shown) revealed that the drug absorption procedure reduced the
Roxifiban concentrations to a level not interfering with the differential ELISA. In addition, DDABs are not absorbed from plasma specimens.

The following changes were included compared to ‘quantification of free DDABs in plasma’ method, including use of less diluted plasma (1/10), and procedures aimed at reducing the background. The latter consisted of IgG depletion of purified GP IIb/IIIa (13) and GP IIb/IIIa antagonist addition in the ‘no-drug’ control wells during the detection step with the secondary antibody. The latter procedure reduced the background readings of some plasmas, thereby increasing the signal to noise of the assay. While the mechanism of background reduction by Roxifiban has not been completely elucidated, it may be related to the dissociation of conformationally sensitive, non drug-dependent antibodies from the ELISA plate.

**Result reporting and quality assurance.** The prospective use of the assay required rapid turnaround of results (24 to 48 hours) for specimen shipped on dry ice from the various clinical sites. Analysis was performed under GLP guidelines at Covance Inc. (Vienna, Virginia). The assay format incorporated both positive and negative controls on all plates. Plate acceptance and rejection criteria were established initially using citrate plasma from a previously described DDAB positive thrombocytopenic patient (patient 330 in 13). Briefly, this patient experienced a thrombocytopenic episode in a previous trial with Roxifiban. This quality control material was replaced during the first clinical study with a renewable source of antibodies (a recombinant chimeric DDAB [rJK094]). Briefly, the antibody was derived using standard hybridoma technology after immunization of mice with purified GP IIb/IIIa / Roxifiban complexes. Resulting hybridomas were screened in the differential ELISA and one clone with differential reactivity was isolated, cloned, and
the constant region was replaced with human IgG<sub>4</sub> (manuscript in preparation). This antibody was used as a positive control after cross-validation with plasma from patient 330. Changes in absorbance after substrate addition were determined kinetically for 5 minutes at 650 nm or until the change in absorbance reached 0.5 OD units. The kinetic reading is insensitive to minor variations in the time of substrate addition to individual wells. In addition, specimen with different background readings (signal in the absence of Roxifiban addition) can be accommodated on the same ELISA plate (13). Results are expressed as delta \[ (V_{\text{max}} \text{ plus compound}) - (V_{\text{max}} \text{ minus compound}) \]. Assays were performed with triplicate determinations and a coefficient of variation around the cut-off (see below) of less than 15% was observed. Limited stability studies revealed that the DDAB concentration was unchanged in samples stored for up to two years. The ELISA results from 108 non-thrombocytopenic subjects treated with Roxifiban (pre-dose samples from a previous phase II Roxifiban study; 13) were used to derive a statistical cut-off value (medical decision level) with a positive rate of 5% (delta=12 for ‘Quantification of free DDABs’ and a 1/20 sample dilution and delta=35 for ‘Quantification of platelet-bound and free DDABs’ and 1/10 sample dilution; 95th percentile of non-parametric rank order test).

Miscellaneous. Platelet absorption studies were performed as described previously (13). For all in vitro studies, XP280, the salt of the active, free acid from of the pro-drug Roxifiban was employed (19).
Results

Frequency of thrombocytopenia in patients treated with Roxifiban in the absence of DDAB testing. A total of 386 patients with CAD or PAD were treated with Roxifiban without DDAB testing (retrospective testing in Table I). Eight cases of thrombocytopenia were observed (Table I). The frequency of thrombocytopenia was not different between CAD (4/203) and PAD (4/175) clinical trials (Table I). The statistical power of the clinical studies was insufficient to determine whether thrombocytopenic patients differed by age, gender, weight, or morbidity and mortality. The overall frequency of thrombocytopenia in the patient group was 2.1%. Half of the cases occurred early (days 2 to 4), whereas the other half presented with delayed (days 11-16) thrombocytopenia. The vulnerable period for thrombocytopenia appears to be the first 3 weeks of treatment. Additional cases were not observed in patients treated for up to 6 month. The extent of thrombocytopenia varied from moderate to severe (less than 20,000 platelets per microliter). In all cases, treatment with Roxifiban was discontinued and patient safety was ensured by close clinical and laboratory monitoring, and hospitalization and platelet infusions if required. None of the thrombocytopenic patients experienced major bleeding episodes at the time of thrombocytopenia and all patients recovered without additional sequelae. Based on the availability of specimens, retrospective testing for DDABs was performed as indicated. Five of six patients tested positive for DDABs. Some patients were already antibody positive prior to treatment, whereas others developed an increased in antibody titer temporally associated with the thrombocytopenic events. These observations suggest that the prevailing
pathomechanism for thrombocytopenia in this patient population is immune-mediated platelet clearance and/or destruction.

**Implementation of testing for free DDABs in a phase II study with a drug holiday.**

With the observation that thrombocytopenia is a relatively common side effect of Roxifiban, we tested the hypothesis that prospective testing for DDAB reduces the frequency of thrombocytopenia. Patients negative by ELISA entered into a qualification period and were treated with Roxifiban on five consecutive days, followed by a ‘drug-holiday’ for 9 days. Additional antibody testing was performed on days 7 and 14. A confirmed positive DDAB result was used as exclusion criteria. The remaining patients entered into a 6-month treatment phase. Platelet counts were taken to further ensure patient safety. 4.3% of patients (57/1332) tested positive at prescreen, 0.5% (4/823 patients) tested positive for DDAB at day 7-8, and an additional 2.2% (18/821) tested positive on day 14. Note that some of the subjects were not enrolled because other study enrollment criteria were not met. During this study, one patient (ECH) tested negative for the presence of DDABs at pre-screen and developed thrombocytopenia on day 14, nine days after discontinuation of drug treatment. The temporal association between increase in antibody concentration and reduction in platelet count is illustrated in Figure 1A. A strong positive DDAB response was detected in the day 14 plasma (Fig. 1A). It should be noted that platelet GP IIb/IIIa serves as a reservoir for Roxifiban and blood concentrations are estimated to be 1-2 nM nine days after discontinuation of treatment. This drug level is sufficient for a maximal response in the DDAB ELISA (13) and predicts an occupancy of 5% of GP IIb/IIIa binding sites in vivo. Thus, these data do not argue against DDABs being causative for thrombocytopenia in the presence of a ‘drug holiday’.
The relationship between the antibody present in plasma and the pathogenetic antibody is suggested by the following observations. Platelet absorption studies revealed that the ELISA positive DDAB bound to gel purified platelets in the presence, but not absence of Roxifiban. Moreover, antibodies could be dissociated from the platelet surface by EDTA treatment, a condition previously reported to disrupt the GPIIb/IIIa complex (not shown).

Re-analysis of the pre-dose sample of patient ECH provided evidence for an inconsistently detectable antibody concentration (3 out of 7 determinations were positive). Additional studies suggested a low affinity DDAB with binding being sensitive to the washing conditions of the ELISA plate. Replacing the automated plate washer with a ‘hand-wash’ enabled consistent detection of this antibody (Fig. 1B). Comparison of serial dilutions of the pre-screen versus day 19 specimen provided evidence for a 20-fold increase in antibody concentration (Fig. 1B). Additional plasma samples were obtained from seven patients that were negative upon prescreen but were excluded from the clinical trial based on a positive antibody concentration (Fig. 1C). No significant change in platelet counts was observed in these patients.

Implementation of testing for free and platelet bound DDABs in a phase II study without drug holiday.

With the aim of capturing free and platelet bound DDABs to Roxifiban in plasma and thereby decreasing the time required to detect a serum-conversion in patients not presenting with pre-existing antibodies, a modified DDAB ELISA was implemented in a second phase II study. The key change is EDTA treatment of platelets to release platelet bound DDABs that recognize complex-specific GP IIb/IIIa epitopes (see Materials and Methods). Patients with negative prescreen were continuously treated up to 180 days and
additional DDAB testing was performed on days 7, 10, and 14. 5.4% of patients were excluded from the study: 3.0% (15/493) patients were excluded from the study based on a prescreen and additional 2.4% (8/342) that tested positive during either days 7-8, day 10, or day 14-15. Some of the prescreened subjects were not enrolled because other study criteria were not met. We observed one case of thrombocytopenia (Fig. 2A). The patient (SGK) tested negative for the presence of DDABs prior to drug treatment and received Roxifiban for 8 days. DDAB analysis of the day 8 sample was positive (delta=90). The physician was notified, the patient hospitalized, and drug treatment terminated. A further rapid increase in DDAB concentration was observed, coinciding with a sharp fall in platelet number (Fig. 2A). Re-analysis of the pre-dose and days 3 and 5 specimen by both the EDTA (closed circles) and citrate (open circles) methodologies failed to detect antibodies at earlier time points (Fig. 2A). Thus, thrombocytopenia was either elicited by a de novo immune response or the sensitivity of the ELISA was insufficient to detect pre-existing antibodies in this patient.

Treatment with Roxifiban was discontinued in 7 additional patients due to a developing DDAB concentration without a significant reduction in platelet count. The time-course of serum-conversion of three representative patients is depicted in figure 2, panel B to D. To test whether the EDTA elution facilitated earlier detection of serum-conversion, specimens were retrospectively analyzed by both methods (Fig. 2A to D). Overall, the time-courses of serum-conversion were very similar between the EDTA and citrate samples, suggesting that the EDTA procedure had little benefit in detecting a rise in antibody concentration at earlier time points due to elution of platelet-bound IgG.
Studies were performed to delineate whether ELISA detectable antibodies have the propensity to be pathogenetic (platelet-binding antibodies) or alternatively, recognize epitopes elicited in GP IIb/IIIa by the immobilization procedure used in the assay procedure. DDAB containing plasma was incubated with platelets in the presence or absence of Roxifiban and re-analyzed in the DDAB ELISA. The results are summarized in Table II. Platelet absorption reduced the ELISA detectable antibody concentration in the presence, but not absence of Roxifiban, indicating the specificity of the platelet absorption. These results indicate that the antibodies have the propensity to bind to intact platelets in a drug-dependent manner. These observations are consistent with the possibility that discontinuation of Roxifiban treatment based on the positive DDAB test may have prevented thrombocytopenia.

**Effects of prospective testing for DDABs on the frequency of thrombocytopenia.**

Thrombocytopenia frequencies were compared between clinical trials as a function of the use of the DDAB ELISA to control patient enrollment. All patients included in the comparison presented with advanced atherosclerotic vascular disease. While different DDAB testing protocols were employed (free DDABs versus free and platelet-bound DDABs), the test results were similar for both test methods (compare free versus free and platelet DDAB time courses in Fig. 2 A-D). The treatment intervals with respect to the ‘drug holiday’ were different between the studies. However, the thrombocytopenia observed during the ‘drug holiday’ (Fig. 1), nine days after discontinuation of treatment, appears to be the result of drug exposure. These considerations suggest that a ‘drug holiday’ was not fully operative. A 2.1% frequency of thrombocytopenia was observed when 386 patients were enrolled without negative
DDAB result as an inclusion criterion. Of the 1044 patients treated after DDAB testing, 2 patients developed thrombocytopenia with an overall frequency was 0.2%. Statistical analysis revealed a highly significant (p=0.0007) reduction of thrombocytopenia when a negative DDAB test was used as an inclusion criterion (Table III), further supporting the immune nature of thrombocytopenia during treatment with Roxifiban. In a subgroup analysis, the statistical evaluation was limited to CAD patients. Despite reduced sample size, the reduction in thrombocytopenia frequency by DDAB testing was still highly significant (p=0.0078). Taken together, these observations suggest that thrombocytopenia during treatment with Roxifiban can be prevented by testing for DDABs.

**Discussion**

Thrombocytopenia during treatment with Roxifiban occurred during two time periods, either early in the first 4 days of treatment (early-onset thrombocytopenia) or within 11-16 days (delayed-onset thrombocytopenia). No additional cases were observed with up to 6 month of Roxifiban treatment. Thus, the critical timeframe for the development of thrombocytopenia during treatment with Roxifiban is the first 3 weeks. The implication of these findings is that patient safety should be ensured by closely monitoring the platelet counts during this time period or potentially, using DDAB testing. However, the clinical utility of DDAB testing needs confirmation in large clinical trials. The results presented here further support the immune nature of thrombocytopenia during treatment with the GP IIb/IIIa antagonist Roxifiban. This conclusion is based on the observation that controlling patient enrollment using a positive DDAB ELISA test result prior to or during treatment as exclusion criteria statistically significantly reduced the
incidence of thrombocytopenia. In addition, with the exception of one patient, DDABs could be demonstrated by retrospective analysis in all patients with thrombocytopenia when specimen were available. It remains unclear whether this patient experienced a non-immune mediated thrombocytopenia or the assay failed to detect this specific antibody. Overall, the frequency of non-immune mediated thrombocytopenia during Roxifiban treatment must be very low.

The medical decision level employed in these studies was based on a statistically-based cut-off level for DDAB concentration derived from non-thrombocytopenic patients of a previous phase II trial of Roxifiban. The clinical studies were not designed to determine the false positive rate of the DDAB ELISA, since patients with positive antibody reaction were excluded from the study. However, some estimates as to the false positive rate can be obtained by comparing clinical trials with and without the prospective use of the DDAB ELISA. For example, between 5% (free and platelet bound DDABs) and 7% (free plasma DDAB) of patients were excluded from the study based on a positive antibody reaction at pre-screen or during treatment. Assuming a thrombocytopenia frequency of two percent, the false positive rate could be as high as 5 percent. This false positive rate would be, in our view, unacceptable for routine clinical use. However, it should be noted that further clinical studies may help to optimize the clinical decision level to reduce the false positive rate. In addition, patients presenting with a positive DDAB test prior to treatment may benefit from more extensive monitoring. The observed false negative rate is low (0.1%). The false negative test result (1 subject) was not due to a non-immune nature of the thrombocytopenia. Further characterization of this plasma provided evidence for low affinity antibodies, and after
assay modifications to efficiently capture these antibodies, the pre-screen was positive. Because of the potential serious sequelae of thrombocytopenia, false positives are preferable over false negatives.

A number of patients developed a positive antibody reaction during treatment with Roxifiban and drug treatment was stopped. Analysis of these time course plasmas from study by platelet depletion (Table II) revealed that the majority of the antibodies bound to platelets. This observation suggest that the patients were at increased risk for thrombocytopenia. Two factors may explain why thrombocytopenia was not observed in these patients. First, DDABs derived during treatment with Roxifiban are exquisitely drug dependent (13) and discontinuation of treatment may have reduced the Roxifiban blood concentration below levels required for sufficient antibody binding to platelets. Second, none of these plasmas presented with antibody concentrations as high as those observed in thrombocytopenic patients (compare Figure 2 and reference 13).

Whole blood was collected from patients presenting with an increase in antibody concentration in either sodium citrate or EDTA to test whether EDTA-dissociation of platelet-bound DDABs that are complex specific alters the time to serum conversion. Generally, we observed an increased DDAB concentration in samples collected in EDTA compared to citrate. However, the increase was modest, and little effect on time to reach the medical decision level was observed. These observations suggest that platelet elution has little advantage and do not justify the additional pre-assay sample processing procedures required. This finding is consistent with the idea that antibody loaded platelets are cleared by the reticulo-endothelial system rather than being retained in the circulation.

All cases of thrombocytopenia observed with Roxifiban fall into two categories.
Early-onset thrombocytopenia occurs within the first 5 days of treatment. We observed 4 cases in the absence of DDAB testing, whereas none was observed in the presence of prospective DDAB testing. The available data is consistent with the notion that early onset thrombocytopenia is immune mediated and that the incidence can be reduced by screening patients for DDAB prior to therapy. Delayed-onset thrombocytopenia occurs within the 2nd or 3rd week of treatment. Of the 6 cases in our Roxifiban trials, three presented with a preexisting antibody concentration, whereas three developed a detectable antibodies during treatment. These observations suggest that a second DDAB test during treatment would be required to maximize the utility of the DDAB testing. We also observed that the time interval between DDAB concentration rise and clinically significant reduction in platelet count varied between patients. Many factors, including ‘bone marrow reserve’, and DDAB concentration, affinity, and binding sites on GP IIb/IIIa are expected to affect the propensity to develop thrombocytopenia. In one instance, the rise in antibody concentration was closely followed by thrombocytopenia and could not be prevented by discontinuation of drug treatment. It should be noted that in this case, the DDAB testing was positive prior to a significant fall in platelet counts and allowed for improved patient management in a hospital setting. All cases of delayed-onset thrombocytopenia could be detected by a second test on day 8 after initiating drug therapy. Thus, a prescreen and a day 8 test appear sufficient to significantly reduce the incidence of thrombocytopenia. It remains to be established whether the clinical experience and conclusions related to patient management presented here can be extended to other chemical classes of GP IIb/IIIa antagonists.
Table I: Thrombocytopenia and DDAB status in clinical trials of Roxifiban

<table>
<thead>
<tr>
<th>Subject</th>
<th>Nadir Platelet count x 10^3 per ul</th>
<th>Day of thrombocytopenia</th>
<th>DDABs prior to treatment</th>
<th>DDABs at time of thrombocytopenia</th>
</tr>
</thead>
<tbody>
<tr>
<td>Retrospective Testing for DDABs</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>304</td>
<td>15</td>
<td>4</td>
<td>No sample</td>
<td>No sample</td>
</tr>
<tr>
<td>503</td>
<td>122</td>
<td>3</td>
<td>No sample</td>
<td>No sample</td>
</tr>
<tr>
<td>10205*</td>
<td>47</td>
<td>3</td>
<td>No</td>
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</tr>
<tr>
<td>10403*</td>
<td>79</td>
<td>2</td>
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<td>307</td>
<td>BQL</td>
<td>11</td>
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<td>330</td>
<td>59</td>
<td>16</td>
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<td>55202*</td>
<td>35</td>
<td>13</td>
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<td>58204*</td>
<td>5</td>
<td>14</td>
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<td>Yes</td>
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<td>99016</td>
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<td>14</td>
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<td>Yes</td>
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<td>21013</td>
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<td>11</td>
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</tr>
<tr>
<td>(SGK)</td>
<td></td>
<td></td>
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<td></td>
</tr>
</tbody>
</table>

Thrombocytopenia is defined as platelet number below 90,000 per microliter or reduction by more than 50% compared to the pre-dose specimen. Nadir: platelet count per microliter during thrombocytopenia. Day: earliest time point thrombocytopenia definition was met. No: DDAB concentration below the medical decision limit. Yes: DDAB concentration above the medical decision limit. Patients 307 and 330 have been
described previously (13). Thrombocytopenia cases during a PAD clinical trial are labeled with *, whereas the remainder of the cases were derived from CAD clinical trials.
Table II: Platelet absorption of ELISA-detectable DDABs

<table>
<thead>
<tr>
<th></th>
<th>Delta (no depletion)</th>
<th>Percentage depletion (minus Roxifiban)</th>
<th>Percentage depletion (plus Roxifiban)</th>
</tr>
</thead>
<tbody>
<tr>
<td>SGK</td>
<td>617</td>
<td>0</td>
<td>77</td>
</tr>
<tr>
<td>JEK</td>
<td>57</td>
<td>0</td>
<td>100</td>
</tr>
<tr>
<td>FD</td>
<td>64</td>
<td>6</td>
<td>93</td>
</tr>
<tr>
<td>EEL</td>
<td>74</td>
<td>15</td>
<td>100</td>
</tr>
</tbody>
</table>

Citrated plasma obtained from the indicated patients was tested by the DDAB ELISA (delta no depletion). Parallel samples were incubated with washed platelets in the absence (minus) or presence (plus) of Roxifiban. Plasma was recovered by centrifugation and analyzed by the DDAB ELISA. Results are expressed as percentage depletion compared to the samples not treated with platelets.
### Table III: Contingency table

<table>
<thead>
<tr>
<th></th>
<th>Thrombocytopenia</th>
<th>No thrombocytopenia</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>No DDAB test</td>
<td>8</td>
<td>378</td>
<td>386</td>
</tr>
<tr>
<td>DDAB test</td>
<td>2</td>
<td>1042</td>
<td>1044</td>
</tr>
<tr>
<td>Total</td>
<td>10</td>
<td>1420</td>
<td>1430</td>
</tr>
</tbody>
</table>

The number of patients treated with Roxifiban without DDAB testing as exclusion criteria is indicated (no DDAB test). After implementation of a DDAB antibody ELISA, patients were excluded from the studies based on a pre-existing or developing DDAB concentration (DDAB test). The frequency of thrombocytopenia in both groups is indicated. The results were analyzed using a two-sided ‘Fisher’s exact test’. A statistically significant reduction in the frequency of thrombocytopenia (p-value = 0.0007) was observed using the DDAB ELISA as an exclusion criteria.
Figure Legends

Figure 1: Time course of DDAB titer development and platelet count.

Patients with a negative DDAB prescreen using citrated plasma were treated with Roxifiban for 5 days (qualification period), followed by a drug holiday for 10 days. During the drug holiday, antibody development was monitored on days 7 and 14.

Panel A: The prescreen citrated plasma for patient ECH was negative for DDABs to Roxifiban. Antibody binding, denoted as delta, is determined by the difference in IgG bound to purified GP IIb/IIIa in the presence of Roxifiban to that in the absence. The patient was treated with Roxifiban for 5 days and developed severe thrombocytopenia on day 14. The time course of DDAB reactivity (closed circles) and decrease in platelet count (closed diamonds) is indicated.

Panel B: Prescreen citrated plasma samples of patient ECH, as well as peak DDAB samples were re-analyzed by the DDAB ELISA. The washing steps with an automated plate washer were replaced by gentle ‘hand-washing’ using a multichannel pipettor. Note that the prescreen of patient ECH is clearly positive using this assay modifications.
Panel C: An increase in antibody concentration was observed in five additional patients (identified by initials). Patients were excluded from the study based on a confirmed positive test result and participated in follow-up antibody testing. The antibody development (delta ELISA) as a function of time (day 1 equals start of qualification period) is indicated. The platelet counts did not change significantly from baseline values.
Figure 2: DDAB development in study DMP 754-017

Patients with a negative DDAB prescreen using an *in vitro* platelet antibody elution technique (EDTA plasma) were treated with Roxifiban continuously for up to 180 days. Additional antibody tests after platelet elution were performed on days 7, 10, and 14.
Drug-treatment in all four subjects was discontinued based on a developing antibody concentration (closed circles). All subjects participated in follow-up antibody testing. In addition, a citrate plasma sample obtained in parallel with the EDTA sample was retrospectively analyzed for the presence of DDABs (open circles). The platelet number is indicated by closed squares.

Panel A: Patient SGK developed severe thrombocytopenia on day 11. Drug treatment was stopped on day 9 based on a rapidly increasing antibody concentration. The patient was hospitalized for monitoring.

Panel B: Patient JEK was excluded from the study on day 11.

Panel C: Patient FD was excluded from the study on day 11.

Panel D: Patient EEL was excluded from the study on day 11.
References


Prospective testing for drug-dependent antibodies reduces the incidence of thrombocytopenia observed with the small molecule glycoprotein IIb/IIIa antagonist Roxifiban: implications for the etiology of thrombocytopenia