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Novel Antiplatelet Agents: ALX-0081, a Nanobody Directed towards von Willebrand Factor

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Abstract This manuscript reviews the studies performed with ALX-0081 (INN: caplacizumab), a Nanobody targeting von Willebrand factor, in the context of current antithrombotic therapy in coronary artery disease. ALX-0081 specifically inhibits platelet adhesion to the vessel wall, and may control platelet aggregation and subsequent clot formation without increasing bleeding risk. A substantial number of antithrombotics are aimed at this cascade; however, their generally indiscriminative mode of action can result in a narrow therapeutic window, defined by the risk for bleeding complications, and thrombotic events. Nonclinically, ALX-0081 compared favorably to several antithrombotics. In Phase I studies in healthy subjects and stable angina patients undergoing percutaneous coronary intervention (PCI), ALX-0081 was well tolerated, and effectively inhibited pharmacodynamic markers. Following these results, a phase II study was initiated in high-risk acute coronary syndrome patients undergoing PCI. Based on its mechanism of action, ALX-0081 is also being developed for acquired thrombotic thrombocytopenic purpura.

Keywords ALX-0081 Nanobody · Caplacizumab · von Willebrand factor · Antithrombotic · Acute coronary syndrome · Percutaneous coronary intervention · Antiplatelet inhibition · Adjunctive pharmacotherapy · Thrombotic thrombocytopenic purpura

[1]Nanobody® is a registered trademark of Ablynx NV

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Introduction

Coronary artery disease (CAD) is one of the leading causes of morbidity and mortality worldwide with clinical manifestations ranging from stable angina to acute coronary syndromes [1]. While the former is associated with stable but progressive atherosclerotic plaque growth within the coronary arterial wall, acute coronary syndromes generally result from endothelial damage, plaque rupture, and subsequent clot formation.

The successive adhesion, activation, and aggregation of platelets are key processes in arterial thrombus formation after endothelial damage. This cascade is partly mediated by von Willebrand Factor (vWF), as suggested by its correlation with impaired endothelial function [2]. Not surprisingly, a substantial number of antithrombotic drugs are aimed at different steps in the platelet activation and aggregation cascade, aiming to balance normal hemostasis with pathological clot formation [3–6].

Optimal pharmacological therapy, and where necessary, percutaneous coronary intervention (PCI), are the cornerstones of nonsurgical CAD management [7]. In patients undergoing elective PCI, standard adjunctive pharmacotherapy consists of heparin and dual antiplatelet inhibition, based on drugs with complementary mechanisms of action. Acetylsalicylic acid is the most widely used antiplatelet drug that irreversibly inhibits the synthesis of thromboxane A_2, a mediator of the glycoprotein GPIIb/IIIa receptor complex [8]. Clopidogrel (Plavix®), prasugrel (Effient®), and ticlopidine (Ticlid®) irreversibly, and ticagrelor (Brilinta®) reversibly inhibit the $P2Y_{12}$ subtype of the platelet adenosine diphosphate receptor, thereby blocking the activation of the glycoprotein GPIIb/IIIa pathway [5, 6, 9]. The treatment of high-risk acute coronary syndrome (ACS) patients undergoing PCI is more aggressive, and may also include abciximab (ReoPro®) on top of the standard antithrombotic regimen [3,

5, 6, 10, 11]. Abciximab, as well as eptifibatide (Integrilin®) and tirofiban (Aggrastat®) are direct inhibitors of the platelet GPIIb/IIIa receptor, and prevent the final, common result of platelet activation, i.e., platelet aggregation, by blocking the binding of fibrinogen and other adhesive ligands to the receptor complex [12].

As the currently available antiplatelet drugs affect the entire cardiovascular system, their use is often associated with a narrow therapeutic window [13, 14]. On the safety side, this window is determined by a dose-limiting risk for bleeding complications, including bleeding at catheterization sites, gastrointestinal bleeding, and cerebral hemorrhage [3, 4, 15]. The timely resolution of these bleeding events can also be hampered by the irreversible mode of action of some antiplatelet drugs. On the ischemic side, resistance to aspirin and/or clopidogrel has been reported in a significant number of patients [3, 4, 15–17]. The latter might be responsible for suboptimal platelet inhibition, exposing patients undergoing PCI to an increased risk of thrombotic events [18]. Therefore, a considerable unmet clinical need in antithrombotic therapy resides in development of a pharmacologic agent that is clinically effective, without compromising safety.

The interaction between platelets and vWF is an attractive upstream target in the thrombotic process (Fig. 1) [19]. vWF is an adhesive plasma glycoprotein with pivotal role in the recruitment of platelets to sites of vascular injury, by bridging between exposed collagen in the damaged vessel wall and the GPIb receptor on the surface of platelets [20]. The reversible binding of GPIb to the A1 domain of collagen-bound vWF allows platelets to roll over the damaged area, which is then followed by firm adhesion through the platelet collagen receptors and eventually platelet activation, fibrinogen binding, platelet aggregation and thrombus formation [20, 21].

Since the vWF A1 domain is only accessible after a conformational change induced by immobilization and high shear conditions in small or stenosed arteries, agents targeting the vWF A1–platelet GPIb interaction could allow for more selective inhibition of the thrombotic process [19, 21]. Indeed, the antithrombotic effect of several compounds targeting this axis has been demonstrated in vitro and in vivo, without increasing bleeding risk [22–27]. Nevertheless, up to now, only two drug candidates have entered clinical evaluation, aside from ALX-0081 (INN: caplacizumab): the therapeutic aptamer ARC1779, and the monoclonal antibody AJW200 [28, 29]. While for the latter, only anecdotal phase I results are available (showing good tolerability in healthy volunteers, without increasing bleeding complications) [28], ARC1779 has been evaluated in several phase II studies in thrombotic indications, including patients undergoing carotid endarterectomy and patients with congenital defects of vWF [29]. Tolerability and clinical efficacy seem promising so far, but similar to ALX-0081, further clinical development is needed to fully establish its therapeutic potential [29].

ALX-0081 is a bivalent Nanobody directed towards the A1 domain of vWF, and specifically inhibits interaction of vWF with the platelet GPIb receptor complex, the first step in platelet adhesion to the vessel wall under high shear conditions (Fig. 1). Nanobodies are a novel class of therapeutic proteins, and are based on the smallest functional fragments of single-chain antibodies that occur naturally in the *Camelidae* family (Fig. 2). They have a high degree of homology (in terms of sequence and structure) to human immunoglobulin heavy chain variable region domains, can be engineered into different formats, and can be expressed by prokaryotic and eukaryotic host cells. Nanobodies are believed to combine the advantages of conventional antibodies with important features of small molecule drugs, and are being developed for a range of diseases (including inflammation, hematology, oncology, and pulmonary disease).

Nonclinical Efficacy and Safety Compared to Currently Marketed Antiplatelet Drugs

The nonclinical development of ALX-0081, as detailed in [30], showed that the Nanobody avidly binds to vWF in vitro, and potently inhibits ristocetin-induced binding of vWF to platelets. Results from in vitro perfusion chamber experiments also confirmed that ALX-0081 selectively inhibits platelet adhesion at high shear rates, such as those observed in normal arterioles and stenotic arteries [30]. The antithrombotic effect of ALX-0081 was further demonstrated in ex vivo perfusion studies, using blood from healthy individuals and PCI patients [30]. While the latter also received standard of care treatment (aspirin, clopidogrel, and unfractionated heparin), this resulted in only partial inhibition of ex vivo platelet adhesion [30]. In contrast, the inhibition was complete after addition of ALX-0081 [30].

The in vivo efficacy of ALX-0081 was studied in a modified Folts model in baboons, mimicking unstable angina [30]. Only ALX-0081 and abciximab were able to completely abolish cyclic flow reductions in this model, even after infusion of epinephrine, a stimulator of platelet activation [30]. Aspirin and heparin were not effective, and clopidogrel only showed a weak antithrombotic effect [30]. The results also indicated that lower doses of ALX-0081 were needed to obtain the same effect as abciximab [30].

The antithrombotic effect of ALX-0081, abciximab, and clopidogrel in the modified Folts model was further evaluated via surgical bleeding, i.e., by measuring mean blood loss from a well-defined incision [30]. Blood loss in animals receiving ALX-0081 was less than in clopidogrel- and

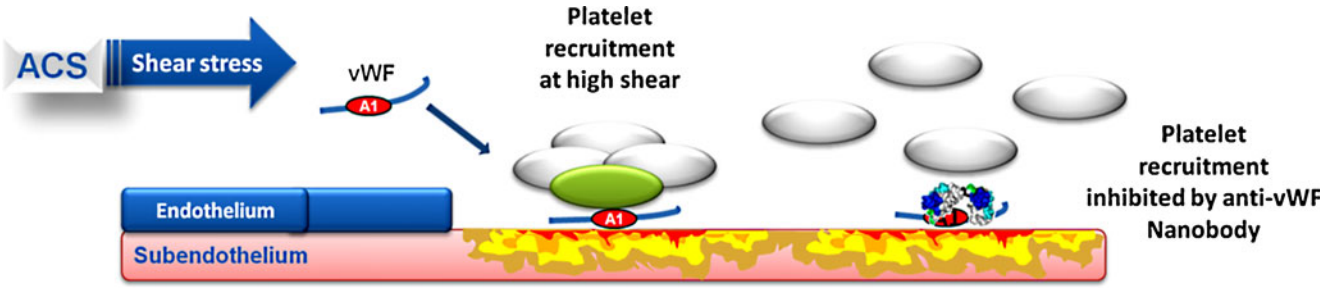

Fig. 1 Inhibition of platelet adhesion by the anti-vWF Nanobody in high shear conditions. Upon vascular damage, the sub-endothelial structure becomes exposed to the blood stream, leading to immobilization of vWF to the subendothelium. Due to immobilization and shear stress in coronary arteries, a conformational change in vWF is induced, activating the vWF A1 domain, which in turn binds to the GPIb receptor of platelets. Without inhibition, the A1 domain will "recruit" platelets, causing platelet adhesion. By inhibiting the A1 domain, vWF-mediated platelet adhesion is prevented

abciximab-treated animals, indicating that ALX-0081 may be safer in terms of bleeding risk, and, combined with the aforementioned findings, could have an increased therapeutic window [30]. Addition of a standard therapeutic combination of heparin, aspirin, and clopidogrel to ALX-0081 did not further enhance efficacy as compared to ALX-0081 monotherapy, but also did not increase surgical bleeding tendency [30].

Throughout the nonclinical studies, ALX-0081 showed favorable PK properties [30]. ALX-0081–vWF complexes stay in circulation with a terminal half-life coinciding with the reported half-life for vWF, and are most likely degraded via a hepatic pathway, similar to free vWF [30]. Excess (unbound) ALX-0081 however is rapidly cleared via the kidneys, unveiling a self-regulated PK mechanism that is expected to contribute to a low overdose potential [30]. In

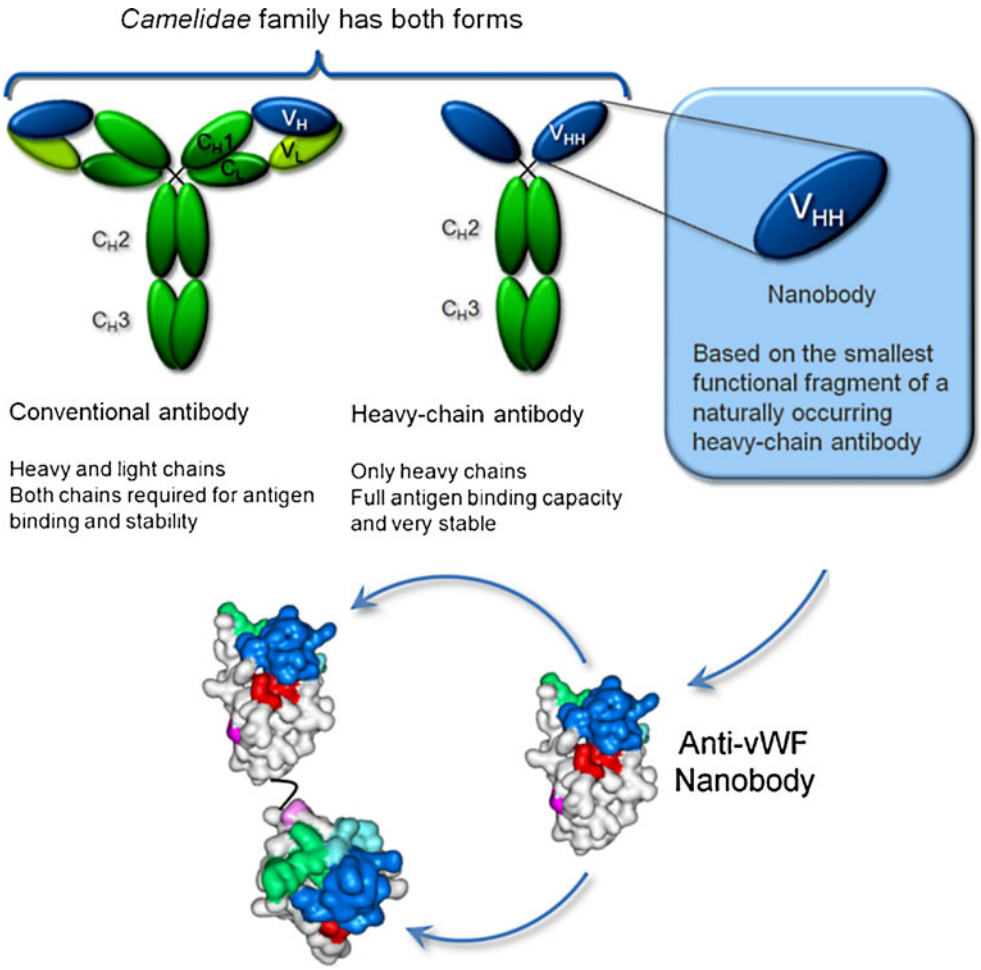

Fig. 2 General description of Nanobodies and ALX-0081. ALX-0081 consists of two identical building blocks (moieties) directed towards vWF, joined by a 3-alanine linker. Courtesy of Jan Steyaert et al. VIB Department of Structural Biology

addition, the action of ALX-0081 can be reversed by administration of vWF [30], suggesting that if necessary, plasma-purified vWF concentrates can be used as an antidote in a clinical setting.

Dose Ranging in Healthy Volunteers

The initial single-center, double-blind, randomized, placebo-controlled study [31] included 40 male subjects, recruited according to standard criteria for first-in-man studies. The primary objective of the study was to evaluate the safety and tolerability of ALX-0081, administered as single i.v. infusion at 0.5, 1, 2, 4, 8, and 12 mg (Table 1). To closely monitor bleeding risk, prothrombin time (PT), activated partial thromboplastin time (aPTT), and vWF and factor VIII (FVIII) levels were assessed throughout the study. Secondary objectives included pharmacokinetics, immunogenicity, and pharmacodynamics, as determined by template bleeding time and the degree of ristocetin-induced platelet aggregation (RIPA).

The administration of single drug infusions did not result in signs of local intolerance. Safety laboratory monitoring did not reveal any ALX-0081-related effects on hematological parameters, clinical chemistry, cardiovascular monitoring, or physical examinations. No serious adverse events or dose-limiting toxicity occurred and the safety analysis indicated that single i.v. infusions of ALX-0081 were well tolerated throughout the dose range. ALX-0081 treatment also had no clinically relevant influence on PT and aPTT.

Decreases in FVIII and vWF antigen (FVIII/Ag and vWF/Ag) levels occurred in all subjects receiving ALX-0081 (Fig. 3). The effects were considered mild and transient, without clear dose relation, and without clinical significance in terms of bleeding. The decreases in both parameters occurred congruently in the same subjects and are believed to be directly linked to the pharmacology of ALX-0081: vWF serves as a carrier for FVIII, and likely has a slightly increased clearance when complexed with ALX-0081.

The kinetic profile revealed nonlinearity and a fast initial elimination phase, followed by a prolonged secondary phase. No signs of immunogenicity were detected up to 30 days after the last administration of ALX-0081. The pharmacodynamic effect of ALX-0081 was assessed by a RIPA assay. To this end, ristocetin was added to plasma samples at a final concentration of 1.5 mg/mL, to induce maximal platelet aggregation. The results showed that the pharmacodynamic active dose for inhibition of vWF-mediated platelet aggregation was reached at 2 mg ALX-0081, as indicated by RIPA levels dropping below 10 % maximal aggregation. For higher doses, the duration of this effect increased. The recovery times of the inhibitory effect varied substantially between subjects, but returned to baseline levels within 24 h in most. Template bleeding time also increased with increasing doses of ALX-0081, but was not stringently correlated with the duration of RIPA inhibition, nor with the effect of ALX-0081 on FVIII and vWF levels. In all cases where bleeding time was prolonged, bleeding stopped with a pressure bandage and tape, and returned to normal at the 12-h time point at the latest.

Table 1 Clinical development of ALX-0081 (INN: caplacizumab) in coronary artery disease

Study identifier [EudraCT code]	Phase	Study Title	Route, dose	# Subjects
ALX-0081-01/06 [2006-006502-28]	Ia	A phase I, double-blind, randomized, placebo-controlled, parallel group study in healthy male volunteers, to investigate the safety, tolerability and pharmacokinetics of ALX-0081, administered intravenously as single ascending doses	i.v. infusion at 0.5, 1, 2, 4, 8 or 12 mg	40 healthy male volunteers
ALX-0081-1.2/08 [2007-007263-24]	Ib	A phase I, double-blind, randomized, placebo-controlled study of ALX-0081 multiple dose administrations in stable angina (SA) patients undergoing percutaneous coronary intervention (PCI)	Stage A: i.v. infusion at 2, 4, 6, or 9 mg	24 SA patients
			Stage B: i.v. infusion at 6 mg, followed by 3 doses of 4 mg every 6 h	
	Ib	Open-label extension to study ALX-0081-1.2/08	Stage C: i.v. bolus injection at 6 mg, followed by 3 doses of 4 mg every 6 h	22 SA patients
ALX-0681-2.1/09 [2009-012206-39]	II	A Phase II, randomized, open-label clinical trial in high risk PCI patients receiving standard antithrombotic treatment plus either ALX-0081 or GPIIb/IIIa inhibitor (ReoPro®) over a period of 24 h	i.v. bolus injection at 6 mg, followed by 3 doses of 4 mg every 6 h	380 high-risk PCI patients

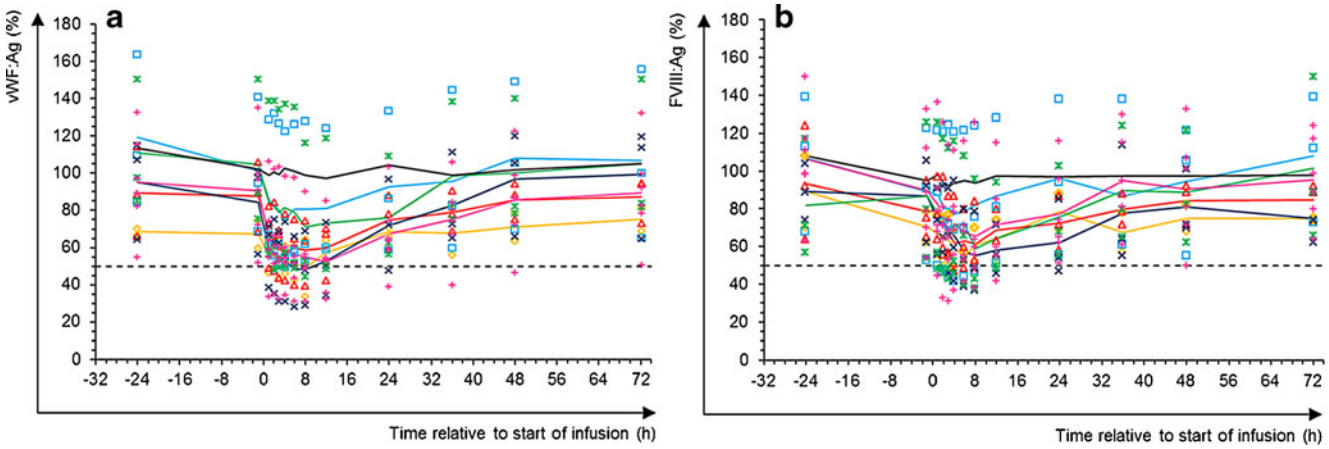

Fig. 3 Pharmacodynamic effect of ALX-0081 on vWF/Ag and FVIII levels. vWF:Ag (**a**) and FVIII:Ag (**b**) levels measured in the phase Ia study in healthy volunteers. Individual data points are depicted by symbols, *colored lines* represent mean per dose level. The placebo mean is indicated by the *black line* (individual data, points not shown). *Horizontal lines* represent the lower limit of normal (50 %). *Orange diamond* 0.5 mg ALX-0081, *red triangle* 1 mg ALX-0081, *aqua square* 2 mg ALX-0081, *blue multiplication symbol* 4 mg ALX-0081, *green asterisk* 8 mg ALX-0081, *pink cross* 12 mg ALX-0081

Phase Ib and Open-Label Extension in Patients with Stable Angina Undergoing Elective PCI

ALX-0081 was further evaluated in a single-center, randomized, placebo-controlled trial in 46 patients with stable angina undergoing elective PCI (Table 1) [32]. All patients received standard therapy consisting of aspirin, clopidogrel, and heparin [33]. The primary objective was to determine the maximum tolerated dose and/or the biologically effective dose. Secondary objectives included the safety and tolerability of escalating doses of ALX-0081, and the biological and clinical response to therapy. Since the antibiotic ristocetin induces binding of vWF to the platelet GPIb receptor, similar to high shear conditions, an automated assay based on ristocetin cofactor (RICO) activity was used as biomarker to assess the biological effectiveness of ALX-0081 [34]. Complete RICO inhibition was defined as less than 20 % maximal aggregation. vWF/Ag and FVIII chromogen (FVIII/C) were used as safety parameters, but also reflect the pharmacodynamic effect of ALX-0081.

The study consisted of three stages. In stage A, ALX-0081 was administered as i.v. infusion at 2, 4, 6, and 9 mg, 60 min prior to the PCI procedure. The dose at which complete RICO inhibition for ≥6 h was obtained, was then explored in stage B (yielding a starting dose of 6 mg, and followed by three subsequent doses of 4 mg every 6 h). After successful completion of stage B and determination of a biologically effective dose, an open-label extension (stage C) was initiated. In stage C, to confirm that the target pharmacological effect could be reached with i.v. injection as well as infusion, ALX-0081 was administered as i.v. bolus with the same dose and administration frequency as in Stage B.

I.v. infusions of ALX-0081 were safe and well tolerated. Increasing the dose in stage A, introducing multiple dosing in stage B, or bolus injections instead of infusions in stage C, did not affect ALX-0081's safety profile. The majority of adverse events were mild and transient, and could be related to the PCI procedure, the underlying disease, or to concomitant medication. The incidence of bleeding was similar in the ALX-0081- and placebo-treated groups, suggesting that ALX-0081 does not increase bleeding risk in this patient population, even when administered on top of a standard antithrombotic regimen. Complementing the safety profile, surrogate efficacy signals showed beneficial effects on indices of coronary microvascular function and endothelial function, as reported in the companion paper [35].

Similar to the phase Ia study, ALX-0081 caused transient decreases in vWF and FVIII levels below 50 %, without clinical significance or symptoms requiring medical attention. Biological activity of ALX-0081, defined by complete inhibition of RICO (<20 % maximal aggregation), was detected in all ALX-0081-treated patients, with a rapid onset (1–6 h after the start of administration) but no clear dose dependency (Fig. 4). In the single-dose cohorts, RICO normalized (>40 % maximal aggregation) between 18 and 48 h post-dose for all but one patient, who showed 37 % aggregation at the last time point (48 h).

After multiple dosing of ALX-0081 in stages B and C, sustained inhibition of vWF-mediated platelet aggregation was observed for up to 30 h post-dosing (Fig. 4). This dose was considered to be the biologically effective dose to be tested in further clinical trials. Five of six patients receiving multiple infusions of ALX-0081 in stage B showed normalized vWF-mediated platelet aggregation within 48–168 h after start of the first infusion. One patient had a RICO level of 35 % at 48 h post-dose, and no further follow-up data were available for this patient. After multiple bolus injections (stage C), 19 of

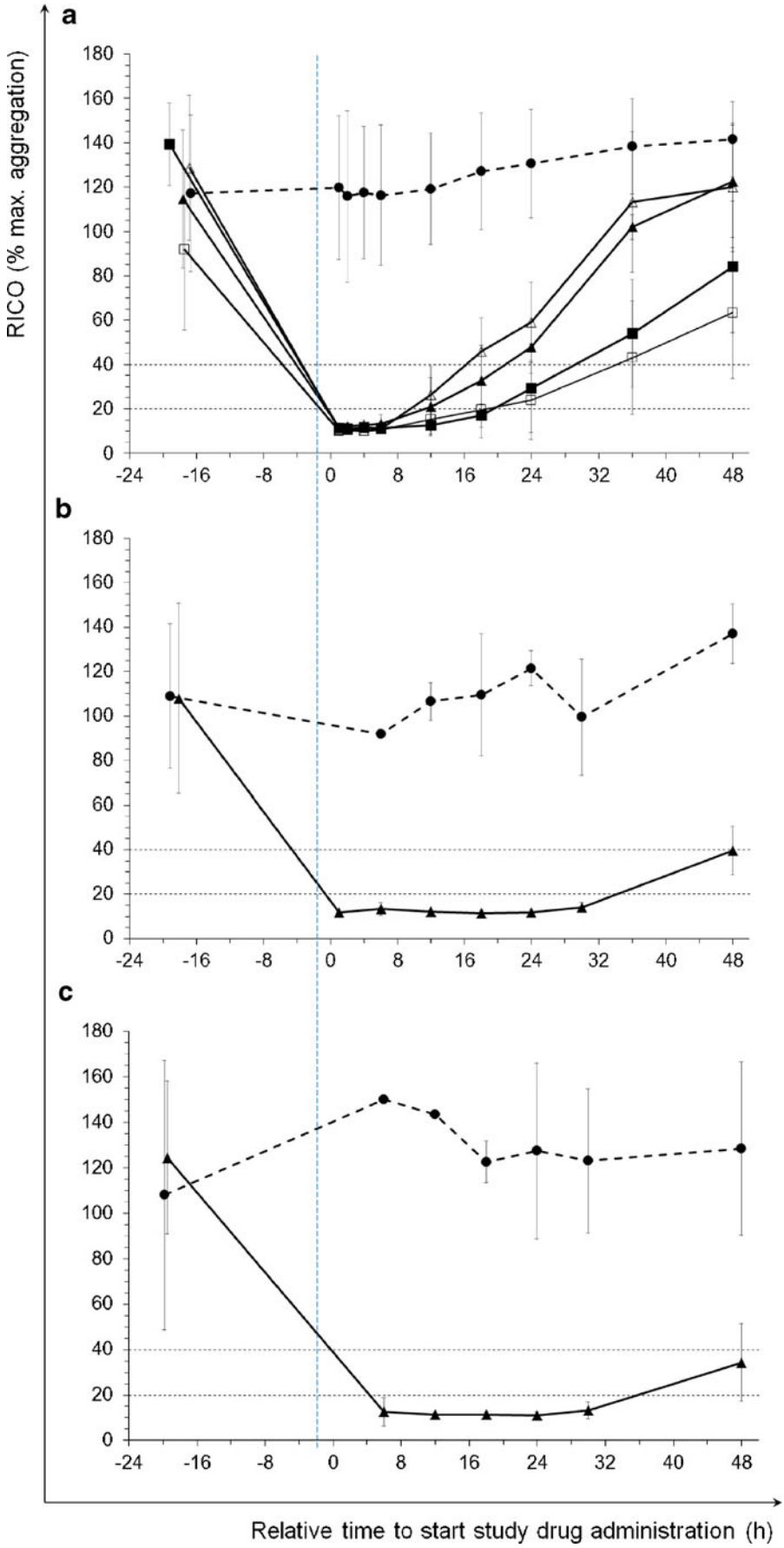

Fig. 4 Pharmacodynamic effect of ALX-0081 on platelet aggregation. Inhibition of vWF-mediated platelet aggregation assessed by RICO (mean±SD per dose level) in the phase Ib and open-label extension study in patients with stable angina undergoing elective PCI. **a** Stage A—single i.v. infusion of placebo or ALX-0081 at 2, 4, 6, and 9 mg, 60 min prior to the PCI procedure. *Dotted line with black circles* placebo, *line with clear triangles* 2 mg ALX-0081, *line with clear squares* 4 mg ALX-0081, *line with black triangles* 6 mg ALX-0081, *line with black squares* 9 mg ALX-0081. The *blue dotted line* represents the time of study drug administration in all three panels. **b** Stage B—multiple 1-h i.v. infusions of placebo or ALX-0081 at a starting dose of 6 mg, followed by three subsequent doses of 4 mg every 6 h. *Dotted line with black circles* placebo, *line with black triangles* 6+4+4+4 mg ALX-0081. **c** Stage C—multiple i.v. bolus injections of placebo or ALX-0081 at a starting dose of 6 mg, followed by three subsequent doses of 4 mg every 6 h. *Dotted line with black circles* placebo, *line with black triangles* 6+4+4+4 mg ALX-0081

20 patients showed normalized vWF-mediated platelet aggregation within 48–168 h after the first injection. One patient had a RICO level of 37 % at 48 h post-dose, and no further follow-up data were available for this patient.

No signs of immunogenicity were detected up to 30 days after the last administration of ALX-0081 in either cohort. PK analysis indicated that exposure increased with dose, and that the terminal half-live was comparable within the investigated dose range. After multiple dosing, steady-state trough concentrations were reached from the second dose onwards, indicating the absence of undue accumulation. This confirmed previous in vivo experiments indicating that unbound ALX-0081 is rapidly cleared, so that only target-bound ALX-0081 is retained in circulation [30].

Phase II Study in High-Risk ACS Patients

Based on the results from the phase I studies, a phase II, randomized, open-label clinical trial was initiated in 380 high-risk ACS patients undergoing PCI (Table 1). The objective of the study is to compare the safety, and more specifically bleeding risk, of ALX-0081 versus the GPIIb/IIIa inhibitor abciximab (ReoPro®) in high risk PCI patients, and to assess tolerability, as well as biological and clinical effectiveness.

The patient population includes adult male and female patients who need to undergo a PCI procedure within 48 h. Patients with unstable angina or non-ST segment elevation myocardial infarction, or stable angina with at least two factors indicating a high risk PCI are eligible. Risk factors for PCI are defined as either patient-related (diabetic patients, renal failure, reduced left ventricular ejection fraction, age, female gender), and/or lesion/anatomy-related (SYNTAX score, bifurcation lesions, multivessel disease, intracoronary thrombus).

Eligible patients are randomized to receive open-label study treatment with either ALX-0081 or abciximab, and are stratified according to PCI procedure type (elective or ad hoc) and stent type (bare metal stent or drug eluting stent). All patients receive the standard antithrombotic regimen of aspirin, clopidogrel, and heparin. ALX-0081 is administered as 6 mg i.v. bolus injection 5–15 min prior to the PCI procedure, and i.v. bolus injections are repeated every 6 h until 18 h post-PCI (3×4 mg), in order to cover a time interval of 24 h peri-PCI. Administration of abciximab consists of an i.v. bolus injection (0.25 mg/kg, administered 10–60 min prior to the PCI procedure), followed by continuous i.v. infusion over 12 h (0.125 μg/kg/min, to a maximum of 10 μg/min).

The primary endpoint of the study is a composite of all bleeding events according to the thrombolysis in myocardial infarction classification, as assessed for all patients within 30 days of study drug administration. The bleeding events are adjudicated by a blinded central endpoint review committee consisting of hematology, cardiology, and clinical pharmacology experts. The primary endpoint is designed to show a 40 % reduction in bleeding events for ALX-0081 compared to abciximab, setting a very high bar to quickly determine the potential of ALX-0081 in the antithrombotic field. Secondary and exploratory endpoints include additional safety parameters, pharmacokinetics, immunogenicity, and pharmacodynamic parameters (RICO, vWF, and FVIII levels).

Future Directions

Considering the narrow therapeutic window of the currently available antiplatelet agents, there is a considerable unmet clinical need in antithrombotic therapy. Since the vWF A1 domain is essential for recruiting platelets and is normally exposed under high shear conditions (i.e., in small or stenosed arteries), agents targeting the GPIb-A1 interaction could allow for more selective inhibition of the thrombotic process. As such, vWF could represent an attractive upstream target for effective antiplatelet agents with improved safety profile.

Nonclinical studies with ALX-0081 (caplacizumab) indicated that the Nanobody could have an increased therapeutic window compared to the currently available agents. The results from the early clinical studies confirmed that ALX-0081 is well-tolerated and does not increase bleeding risk, even when administered on top of a standard antithrombotic regimen. Throughout the clinical studies conducted with ALX-0081, its pharmacodynamics effects were confirmed by a number of biomarkers, and a favorable, self-regulated PK profile was reported. This justifies further phase II studies to address safety and efficacy in patients with ACS.

In the initial stages of clinical development, the safety and efficacy of ALX-0081 is assessed in high-risk ACS patients undergoing PCI. In this setting, ALX-0081 is administered on top of standard antithrombotic treatment (i.e., heparin, aspirin, and clopidogrel, targeting different pathways in the platelet aggregation cascade) [33, 36]. Further stages of development could also assess combinations of ALX-0081 with more recent antithrombotic regimens (incorporating for instance prasugrel or ticagrelor, or the direct thrombin inhibitor bivalirudin). Similarly, platelet reactivity/resistance after clodidogrel and aspirin treatment still needs to be investigated.

On a final note, besides establishing its role in the treatment of ACS, further data will also provide useful information to support development of ALX-0081 for the treatment of acquired thrombotic thrombocytopenic purpura, a rare thrombotic microangiopathy in which deficient vWF processing is heavily implicated [37].

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Ethical standards All studies mentioned in this manuscript complied with the current laws of the country in which they were performed. All clinical studies were conducted in compliance with the principles of ICH and GCP, and the applicable regulatory requirements.

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