Clin Drug Investig (2014) 34:807–818
DOI 10.1007/s40261-014-0236-8

ORIGINAL RESEARCH ARTICLE

A New Reversible and Potent $P2Y_{12}$ Receptor Antagonist (ACT-246475): Tolerability, Pharmacokinetics, and Pharmacodynamics in a First-in-Man Trial

Daniela Baldoni · Shirin Bruderer · Andreas Krause · Marcello Gutierrez · Pierre Gueret · Béatrice Astruc · Jasper Dingemanse

Published online: 21 October 2014

Abstract

Background and objectives ACT-246475 is a new reversible, selective, and potent antagonist of the platelet $P2Y_{12}$ receptor. This study was a first-in-man trial investigating the tolerability, pharmacokinetics, and pharmacodynamics of single oral doses of ACT-246475 and its di-ester prodrug (ACT-281959) in healthy males.

Methods The study had a double-blind, randomized, ascending single-dose design with an oral formulation F1 (i.e., ACT-281959 or placebo) (Part I) and an open-label, randomized, 3-period, crossover design comparing exploratory formulations of ACT-281959 (F2) 70 mg and ACT-246475 (dF) 50 mg to F1 70 mg (Part II). In Part I, doses up to 1,000 mg were tested in 40 healthy subjects. Nine healthy subjects were enrolled in Part II. Standard safety parameters, inhibition of platelet aggregation, and ACT-246475 plasma concentrations were measured. Non-compartmental pharmacokinetic analysis was performed.

Results All doses and formulations were well tolerated. The most frequent adverse event was headache, whereas no events of bleeding or dyspnea were reported. In Part I, ACT-246475 area under the plasma concentration-time curve (AUC) increased dose-proportionally whereas maximum plasma concentration (C_{max}) was less than dose-proportional. The highest C_{max} [geometric mean (95 % CI)] at 1,000 mg was 13.8 (9.7, 19.5) pmol/mL at 4.5 h post-dose, terminal half-life ($t_{½}$) was ~10 h. ACT–246475 C_{max} and $AUC_{0-\infty}$ ratios of geometric means (90 % CI) using F1 as reference, for F2 were 8.5 (5.42, 13.35) and 3.4 (2.40, 4.82), respectively, and for dF 2.2 (1.42, 3.49) and 1.5 (1.07, 2.16), respectively. Mean peak platelet inhibition was 31.0 % after F1 (1,000 mg) and 47.8 % after F2.

Conclusions Oral doses of ACT-281959 and ACT-246475 were well tolerated. Platelet inhibition correlated with ACT-246475 exposure. Exploratory formulations enhanced the bioavailability and antiplatelet effect of ACT-246475.

Key Points

ACT-246475 is a reversible, selective, and potent inhibitor of the platelet $P2Y_{12}$ receptor, well tolerated in healthy male subjects after single doses up to 1,000 mg.

ACT-246475 induced a concentration-dependent inhibition of platelet aggregation, revealed by ex vivo measurements and pharmacokinetic-pharmacodynamic analysis.

1 Introduction

Dual antiplatelet therapy with acetylsalicylic acid (Aspirin®) and a selective antagonist of the $P2Y_{12}$ platelet receptor is the first-line pharmacological therapy recommended for treatment of adverse atherothrombotic events

D. Baldoni (✉) · S. Bruderer · A. Krause · M. Gutierrez · J. Dingemanse
Department of Clinical Pharmacology, Actelion Pharmaceuticals Ltd, Gewerbestrasse 16, 4123 Allschwil, Switzerland
e-mail: daniela.baldoni@actelion.com

P. Gueret
Hemostasis Unit, University Hospital Pontchaillou, Rennes and GETBO EA 3878, Brest, France

B. Astruc
Biotrial, Rennes, France

such as acute coronary syndromes (ACS) [1–7]. $P2Y_1$ and $P2Y_{12}$ are G protein-coupled platelet receptors (GPCRs), activated by the binding to adenosine di-phosphate (ADP). ADP is released at the site of vascular damage and atherosclerotic plaque disruption together with thromboxane A_2 and serotonin and thrombin is generated, to induce the formation of a hemostatic platelet clot and preventing bleeding [8]. Whereas $P2Y_1$ is important for the initial platelet activation and aggregation, $P2Y_{12}$ plays a major role in sustaining irreversible aggregation, leading to thrombus growth in pathological conditions [8–10].

Clopidogrel and prasugrel (both thienopyridines) are inactive prodrugs, and after metabolic conversion into their active moiety, irreversibly bind and inactivate the $P2Y_{12}$ platelet receptor [11–16].

Ticagrelor is a recently approved, non-thienopyridine, reversible $P2Y_{12}$ receptor antagonist, with a fast onset of action [17]. However, both thienopyridines and ticagrelor at higher doses and at higher levels of inhibition of platelet aggregation have revealed increased risk of bleeding and morbidity following bleeding events [11, 13, 15–17].

Therefore, the need of a potent and reversible antiplatelet agent with fast onset of action, low variability of response, and an improved safety profile compared to thienopyridines and ticagrelor remains unmet [9].

ACT-246475 is a new, potent, reversible, and selective antagonist of the platelet $P2Y_{12}$ receptor. ACT-281959 (molecular weight 850.9 g/mol), the di-ester prodrug of ACT-246475 (molecular weight 618.6 g/mol), was developed to improve absorption after oral dosing and is rapidly converted by esterases in vivo to ACT-246475 in two-steps via the formation of ACT-409100 (molecular weight 734.7 g/mol), the mono-ester prodrug (Fig. 1) [18]. Preclinical studies with ACT-246475 in animal thrombosis models demonstrated an improved therapeutic window compared to current available therapies (unpublished data).

This study (ClinTrials.gov NCT01954615) was divided into 2 parts. In the first part (Part I), the safety, tolerability, pharmacokinetics, and pharmacodynamics following single ascending oral doses of an ACT-281959 liquid (emulsion) formulation (F1) were investigated. In the second part (Part II) of the study, the exposure to ACT-246475 after single doses of an exploratory liquid (solution) formulation of ACT-281959 (F2) and a liquid (solution) of ACT-246475 (dF) were investigated and compared to F1.

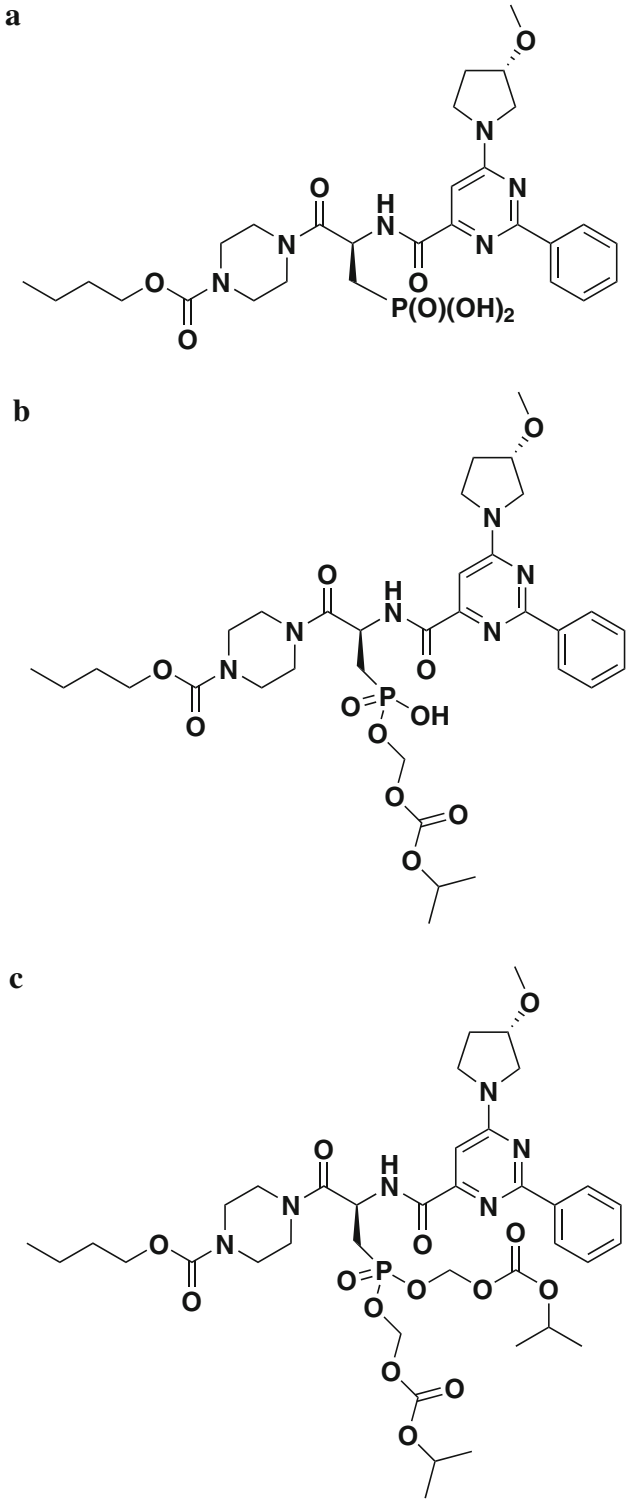

Fig. 1 Chemical structures of: **a** ACT-246475 (active drug), **b** ACT-409100 (mono-ester prodrug), and **c** ACT-281959 (di-ester prodrug)

2 Methods

2.1 Subjects

Non-smoking healthy male subjects between 18 and 45 years of age, with a body mass index between 18 and 32 kg/m^2 were included in the study. Eligible subjects had at screening vital signs, clinical laboratory, and 12-lead electrocardiogram (ECG) results within the normal range and negative results for drug and alcohol use. Subjects

were not eligible if they had any reported family or personal history of bleeding disorders. Previous treatment with acetylsalicylate, non-steroidal anti-inflammatory drugs, or any medication with blood-thinning activity within 2 weeks prior to administration of the study drug was not permitted.

2.2 Study Design

Part I of the study had a double-blind, randomized, placebo-controlled, sequential, ascending single oral dose design during which F1 was tested (emulsion of ACT-281959 or placebo). Five dose groups were treated, each consisting of 6 subjects receiving ACT-281959 and 2 receiving placebo. The sample size of each dose group was based on empirical considerations. In the first two dose cohorts, 5 mg and 20 mg doses were tested. The subsequent dose levels were selected on the basis of an interim analysis, using constraints of a maximum 4-fold dose escalation between successive groups. The doses tested for subsequent groups were 80, 320, and 1,000 mg.

Part II was an open-label, randomized, three-period, crossover, single oral dose study, comparing an oral solution formulation of ACT-281959 (F2) and an oral solution formulation of ACT-246475 (dF) (the active moiety) to the oral liquid emulsion formulation of ACT-281959 (F1). A new group of 9 healthy male subjects attended 3 treatment periods, each separated by a 7-day washout period, randomized to receive the treatments in one of the following 3 sequences: dF/F1/F2, F1/F2/dF, or F2/dF/F1. This ensured the equal distribution of subjects (3 each) within treatment sequences and the equal distribution of treatments in each period. The sample size of each dose group was based on empirical considerations. The doses of ACT-281959 and ACT-246475 selected in Part II were similar upon molecular weight correction: 70 mg ACT-281959 (82.3 mmol) as F1 and F2, and 50 mg ACT-246475 as dF (76.3 mmol of the chloride salt).

In both parts of the study, subjects attended a screening visit within 3 weeks from treatment start. The subjects were admitted to the clinical unit on the day before dosing and remained at the clinic up to 72 h (up to Day 4). An End-of-Study (EOS) visit was performed on the morning of Day 4 (of the third treatment period for Part II). The study was conducted in a Phase I unit (Biotrial, Rennes, France).

The study drugs were ACT-281959, provided as a lyophilisate, and ACT-246475, provided as powder (hydrochloride salt), in sealed glass bottles.

During Part I of the study, ACT-281959 lyophilisate was reconstituted with Lauroglycol^{TM}90 (Gattefossé, Saint-Priest, France) and flavored Methocel^{TM} (Dow Wolff Cellulosics, Bomlitz, Germany) mixed together to obtain an emulsion for oral administration (F1). The concentration of ACT-281959 in F1 after reconstitution was either 1 mg/mL (used in the 5 mg dose group) or 5 mg/mL (used for the 20–1,000 mg dose groups). Placebo matching F1 consisted only of the reconstitution vehicles mixture.

In Part II, F2 was prepared by reconstituting ACT-281959 with: Cremophor® EL (BASF SE, Limburgerhof, Germany)/polyethylene glycol 400/propylene glycol, and a flavored aqueous vehicle mixed together to obtain a solution for oral administration. The concentration of ACT-281959 in F2 after reconstitution was 0.4 mg/mL. Finally, dF was prepared by reconstituting ACT-246475 powder in 100 mL of a pH 7.4 phosphate buffer solution, which gave a solution for oral administration. The concentration of ACT-246475 in dF after reconstitution was 10 mg/mL.

2.3 Safety and Tolerability Evaluations

Safety and tolerability assessments comprised records of adverse events (AEs), serious AEs (SAEs), vital signs, ECG, and laboratory parameters at screening, on the day of treatment start shortly before dosing, and during 72 h post-dose. An AE was any adverse change from the subject's baseline condition, i.e., any unfavorable and unintended sign, including an abnormal laboratory finding, symptom or disease, that occurred during the course of the study, whether or not considered related to the study drug. An AE was considered as SAE, as defined by the International Conference of Harmonization, if fatal, life-threatening, required subject's hospitalization, resulted in persistent or significant disability or incapacity, resulted in a congenital anomaly or birth defect, and/or was medically significant, requiring intervention to prevent at least one of the outcomes listed before.

2.4 Pharmacokinetic Assessments

Blood samples were collected in K_3EDTA tubes containing 10 % solution of dichlorvos (final concentration of 0.01 % per sample) at pre-dose, and 0.5, 1, 1.5, 2, 3, 4, 5, 6, 8, 12, 16, 24, 48, and 72 h post-dose. Dichlorvos served to inhibit blood esterases from hydrolyzing ACT-281959 and ACT-409100 [19]. Within 30 min of collection, the blood samples were centrifuged at approximately 3,000*g* for 10 min at 4 °C. Plasma was transferred and stored at −70 °C.

2.5 Bioanalysis

Validated high-performance liquid chromatography-tandem mass spectrometry (HPLC-MS/MS) assays specific for measurement of ACT-281959 (di-ester prodrug), ACT-409100 (mono-ester prodrug), and ACT–246475 (active drug) concentrations in human plasma were used. During the validation of the analytical method, precision and accuracy, limit of quantification, selectivity, specificity,

recovery, carry-over, linearity, and sample stability were assessed. The validation of the analytical method complied with regulatory guidelines [20, 21].

MS/MS detection (TSQ Quantum, Thermo Fisher Scientific, Waltham, MA, US) analysis was performed in positive ionization mode. The lower limit of quantification (LLOQ) was 1.00 ng/mL for all analytes (1.18, 1.36, and 1.62 pmol/mL for ACT-281959, ACT-409100, and ACT–246475, respectively). For the analysis, the thawed samples were mixed with a solution of deuterated analogs of ACT-281959, ACT-409100, and ACT–246475 (internal standards), and centrifuged for 20 min at approximately 20,200*g* at 8 °C. An aliquot of the worked-up samples was injected and loaded to the HPLC trapping column (XTerra MS C8, Waters, Milford, MA, US) and then eluted to the main column (XTerra MS C18, Waters, Milford, MA, US) for separation and detection by MS/MS. An elution gradient was used with mobile phases A (10 mM NH_4HCO_2, pH 9.0/NH_4OH) and B (NH_4HCO_2 in H_2O/CH_3CN 10:90, v/v, pH 9.0/ NH_4OH).

Inter-run accuracy of quality samples ranged between 105–109 % for ACT-281959, 101–105 % for ACT-409100, and 100–107 % for ACT-246475, inter-run precisions between 4.6–5.6 % for ACT-281959, 4.1–6.5 % for ACT-409100, and 3.3–7.3 % for ACT-246475. Stability was confirmed in plasma up to 6 months of storage at −20 °C or below, for all analytes. The method was selective for each of the analytes, i.e., at each of the retention times, the signal of any interfering peak in matrix blank samples did not exceed 10.0 % of the signal of the lowest concentration samples of the calibration curve.

2.6 Pharmacokinetic Analysis

Non-compartmental pharmacokinetic analysis was performed to determine maximum plasma concentration (C_{max}), time to reach C_{max} (t_{max}), area under the plasma concentration-time curve (AUC) from time zero to the last measurable concentration (AUC_{last}), AUC from time zero to infinity ($AUC_{0-\infty}$) and terminal half-life ($t_{½}$) for ACT-281959, ACT-409100, and ACT-246475 using WinNonLin® 6.1 Software (Pharsight, Mountain View, CA, USA).

Dose proportionality was assessed across the dose range for C_{max} and AUC using the power model [22]. C_{max} and AUC were assumed to be log-normally distributed [23]. Dose proportionality was established if the 90 % confidence interval (CI) of the slope was completely contained in the range (Eq. 1):

$$1 + \frac{\log(0.5)}{\log(r)}, \quad 1 + \frac{\log(2)}{\log(r)} \tag{1}$$

where r denotes the ratio of the highest to the lowest dose. Differences in C_{max}, AUC_{last}, $AUC_{0-\infty}$, and $t_{½}$ between F1 and F2 and between F1 and dF were explored using the ratio of the geometric means and the 90 % CI, with F1 being the reference treatment, derived by a linear mixed-effect model with study treatment and treatment sequence as fixed effects and subject as random effect. Differences for t_{max} were explored using the respective median differences across dose groups and formulations and the corresponding 90 % CI. In Part II, the difference in dose of ACT-281959 and ACT-246475 (82.3 and 76.3 mmol, respectively) was considered small and thus no dose adjustment was done.

The Statistical Analysis System (SAS®) software, Version 9.2 (SAS Institute, Cary, NC, USA) was used for statistical analysis.

2.7 Pharmacodynamic Assessments

ADP-induced platelet aggregation was measured ex vivo during 24-h post-dose. Two methods were applied, LTA (reference gold standard) and VerifyNow $P2Y_{12}$ (novel point-of-care) [24]. LTA was performed with an 8-channel BioData PAP 8E platelet aggregometer (BioData Corp., Horsham, Pennsylvania, USA) in platelet-rich plasma (PRP) (240 µL) and 20 µM ADP in tyrode buffer (10 µL). Blood samples were centrifuged at 230*g* for 10 min at room temperature, the top layer was PRP and was transferred to a second tube. The remaining blood was centrifuged at 1,800*g* for 10 min at room temperature. The top layer was platelet-poor plasma (PPP) and was transferred to a third tube. Platelet count in PRP was measured and adjusted to 260 G/L by dilution with PPP, when required.

VerifyNow $P2Y_{12}$ (Accumetrics, San Diego, CA, USA) assay was performed according to the manufacturer instructions.

Blood samples were collected pre-dose and at 1, 2, 3, 4, 8, 12, and 24 h post-dose.

In Part I, pharmacodynamic samples were collected in 3.2 % (0.109 M) trisodium citrate and were kept at room temperature until analysis.

In Part II, pharmacodynamic sampling was performed after F2 administration only. Samples were collected both in 3.2 % trisodium citrate and in napsagatran (100 µM). Citrate is the commonly used anticoagulant for LTA and VerifyNow $P2Y_{12}$ measurements. By chelating calcium, citrate reduces the free calcium levels in the samples and interferes with the platelet aggregation processes. Napsagatran is a direct thrombin inhibitor and prevents blood coagulation without affecting free calcium levels [25, 26].

2.8 Pharmacodynamic Analysis

The percent change of aggregation from baseline of the MPA (LTA assay) and $P2Y_{12}$ reaction units (PRU) (VerifyNow $P2Y_{12}$ device), per subject and time point were calculated according to Eq. 2:

$$\%\mathrm{IPA}(X)_t = 100 \times \frac{(X_0 - X_t)}{X_0} \quad (2)$$

where $\%\mathrm{IPA}(X)_t$ represents %IPA (percent inhibition of platelet aggregation) derived from the pharmacodynamic variable X (X = MPA or PRU) at time t post-dose (%IPA(M) and $\%\mathrm{IPA}_{\mathrm{PRU}}$, respectively). X_0 and X_t are pharmacodynamic measurements at baseline and time t post-dose, respectively.

The inter-run precision measured during method validation, either in citrate or in napsagatran blood samples, was <20 % for LTA and <10 % for VerifyNow $P2Y_{12}$. The in vitro IC_{50} was derived by spiking blank blood samples collected from healthy subjects with ACT-246475 and was for %IPA(M) 42.7 pmol/mL (citrate blood samples) and 13.8 pmol/mL (napsagatran blood samples) and for $\%\mathrm{IPA}_{\mathrm{PRU}}$ 41.9 pmol/mL (measured in citrate blood samples only) (Eq. 2). The Spearman correlation coefficient for %IPA(M) vs. $\%\mathrm{IPA}_{\mathrm{PRU}}$ in citrate samples was 0.81 ($P < 0.001$).

Summary statistics for the pharmacodynamic variables were calculated using the SAS® software, Version 9.2.

Relationships between ACT-246475 concentrations and %IPA(M) and $\%\mathrm{IPA}_{\mathrm{PRU}}$ variables were estimated based on the (sigmoid) $I_{\max}$ model (Eq. 3) carried out using the software WinBUGS [27] and R Version 2.14.1:

$$\%IPA = I_{\mathrm{placebo}} + \frac{I_{\max} \cdot \mathrm{conc}^{\gamma}}{\mathrm{IC}_{50}^{\gamma} + \mathrm{conc}^{\gamma}} + \varepsilon \quad (3)$$

I_{placebo} inhibition associated with placebo, $I_{\max}$ maximum inhibition, IC_{50} drug concentration associated with 50 % of the maximum inhibition, γ shape/steepness factor, ε error term $\sim N(0, \sigma^2)$, N normal distribution, σ^2 variance associated with error distribution.

3 Results

3.1 Subjects

A total of 49 subjects, 40 in Part I and 9 in Part II, participated in the study and completed the study according to the protocol. Of those, 47 were Caucasians, 1 black, and 1 mixed race, between 18 and 45 years of age.

The overall mean ± SD age, weight, and body mass index (BMI) in Part I were 36 ± 7.1 years, 76 ± 9.4 kg, and 24 ± 2.1 kg/m^2, respectively, and in Part II 30 ± 6.1 years, 73 ± 10.8 kg, and 23 ± 2.3 kg/m^2, respectively. The dose groups in Part I and the treatment sequences in Part II were well balanced with regard to demographic and baseline characteristics.

3.2 Safety and Tolerability

Study treatments were well tolerated. No clinically relevant effects of treatment on the clinical laboratory parameters, ECG recordings, physical examination, and vital sign values were observed.

Altogether, 6 of the 49 subjects reported AEs after dosing. All 6 subjects reported AEs that belonged to nervous system disorders (headache), and 1 of the subjects reported a second AE that belonged to gastrointestinal disorders (nausea). No bleeding events or dyspnea were observed.

In Part I, 4 subjects (13.3 %) reported AEs after administration of ACT-281959 and 1 subject (10.0 %) reported headache and nausea after administration of placebo. In Part II, 1 subject reported 1 AE of headache in each treatment period (11.1 %). Investigational study drug-related AEs were reported between ~1.5 h and ~5.5 h post-dose in Part I and ~6.5 h and ~7.5 h post-dose in Part II. All of the reported events were of mild intensity except for 1 event of moderate headache in 1 subject who had received placebo treatment (F1). At the end of the study, all events were resolved without sequelae.

3.3 Pharmacokinetics

In Part I, all and most of the plasma concentrations of ACT-281959 and ACT-409100, respectively, were below the LLOQ (BLQ). The highest concentration of ACT-409100 was 7.0 pmol/mL measured at 5 h post-dose in 1 subject in the 1,000 mg dose group, with no detectable concentrations later than 12 h post-dose.

The plasma concentration-time profiles of ACT-246475 are displayed in Fig. 2. In the 5 and 20 mg dose groups, all and most plasma concentrations of ACT-246475, respectively, were BLQ. Therefore, non-compartmental pharmacokinetic analysis was performed for ACT–246475 only in the 80 mg (3 out of 6 subjects), 320 mg (5 out of 6 subjects), and 1,000 mg (5 out of 6 subjects) dose groups (Table 1). $C_{\max}$ values [geometric means (95 % CI)] of ACT-246475 in the 80 and 1,000 mg groups were 4.0 (2.9, 5.7) pmol/mL at 1.8 h post-dose and 13.8 (9.7, 19.5) pmol/mL at 4.5 h post-dose, respectively, and $AUC_{0-\infty}$ 18.6 (14.6, 23.7) h·pmol/mL and 194 (155.9, 241.1) h·pmol/mL, respectively. The elimination rate was lower at higher doses, with $t_{½}$ ~2.4 h in the 80 mg dose group, ~3.9 h in the 320 mg group, and ~9.9 h in the 1,000 mg group. Dose proportionality was tested for doses between 80 and 1,000 mg and was to be concluded if the 90 % CI for the population mean slope was contained in the range 0.73–1.27 according to Eq. (1). The 90 % CI for the population mean slope of $C_{\max}$, AUC_{last}, and $AUC_{0-\infty}$ was (0.34, 0.63), (0.82, 1.11), and (0.75, 1.14), respectively.

In Part II, following single oral administration of 70 mg ACT-281959 as F1 and F2, all plasma samples were BLQ for ACT-281959. ACT-409100 plasma concentrations were BLQ in most of the samples after F1. After administration of F2, the highest individual ACT-409100

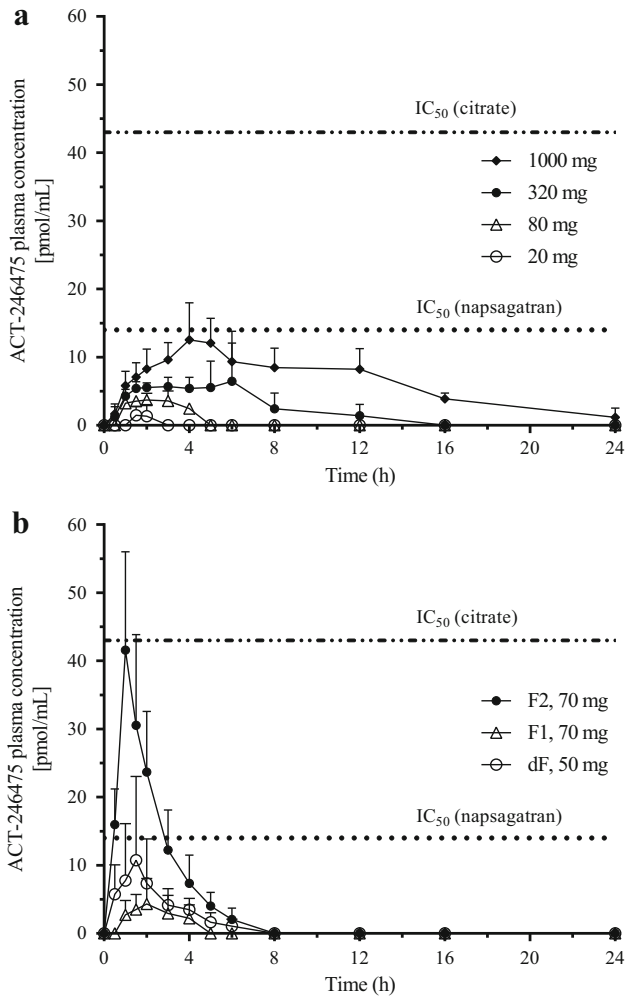

Fig. 2 Mean (SD) plasma concentration-time profiles of ACT-246475 after single oral administration of: **a** 20, 80, 320, and 1,000 mg ACT-281959 as F1 and **b** F1 (70 mg ACT-281959), F2 (70 mg ACT-281959), and dF (50 mg ACT-246475), in healthy male subjects, under fasted conditions. Mean (SD) concentrations were determined for time points at which at least 50 % of values were above the LLOQ. No mean concentrations were calculated for time points later than 24 h post-dose. *Dashed* and *dashed/dotted* reference lines indicate the IC_{50} for %IPA(M) measured during the pharmacodynamic method validation in citrate and napsagatran, respectively. *SD* Standard deviation, *IC50* inhibitor concentration at half-maximal inhibition, *MPA* maximal platelet aggregation

concentration measured was 50.0 pmol/mL at 0.5 h post-dose, with no detectable concentrations in most of the subjects from 2 h post-dose.

Non-compartmental pharmacokinetic analysis of ACT-246475 was performed in 5 of 9 subjects after F1 and in all subjects after dF and F2 (Table 1). Higher plasma concentrations and exposure were observed after administration of F2 and dF than after F1 (Fig. 2; Table 1).

ACT–246475 C_{max} and $AUC_{0-\infty}$ ratios of geometric means (90 % CI) using F1 as reference, for F2 were 8.5 (5.42, 13.35) and 3.4 (2.40, 4.82), respectively, and for dF 2.2 (1.42, 3.49) and 1.5 (1.07, 2.16), respectively. Peak concentrations of ACT-246475 in plasma were reached after 1 h for F2 and dF, and after 2 h for F1. The elimination phase was characterized by a $t_{½}$ of ~2.2, ~1.3, and ~3.2 h after F1, F2, and dF, respectively.

3.4 Pharmacodynamics

In Part I, arithmetic means %IPA(M) and $\%IPA_{PRU}$ (Eq. 2), derived by both LTA and VerifyNow $P2Y_{12}$ up to doses of 320 mg of ACT-281959 (F1) were mostly below 20 % (i.e., the assay inter-subject variability) during 24 h after dosing. After the 1,000 mg dose, the mean %IPA values were above 20 % approximately between 2–3 h and 12 h post-dose. At doses of 80, 320, and 1,000 mg, mean (SD) peak values of %IPA(M) were observed between 2 and 4 h post-dose and were 4.5 % (9.5 %), 17.5 % (13.4 %), and 31.0 % (29.5 %), respectively. The mean (SD) peak values of $\%IPA_{PRU}$ at doses of 80, 320, and 1,000 mg were 13.7 % (6.0 %), 14.2 % (4.9 %), and 32.4 % (8.0 %), respectively, also between 2 to 3 h post-dose. In the placebo group, the highest mean (SD) %IPA(M) and $\%IPA_{PRU}$ were −1.1 % (7.3 %) and 2.1 % (8.4 %), respectively (Fig. 3). The highest individual %IPA(M) and $\%IPA_{PRU}$ measured were 74.3 and 45.2 %, respectively, in the 1,000 mg dose group.

During Part II, arithmetic mean %IPA(M)- and $\%IPA_{PRU}$-time profiles, after F2, showed a peak at 1 h post-dose, with mean (SD) values in citrate of 47.8 (21.3) % and 43.1 (14.8) %, respectively, and in napsagatran of 80.8 (9.5) % and 53.5 (25.6) %, respectively. Peak platelet inhibition, in citrate samples, was followed by a rapid decline to baseline values, with %IPA being ≤20 % from approximately 4 h post-dose. In contrast, %IPA(M) measured in napsagatran remained above 50 % for ~6 h post-dose (Fig. 3). The highest individual %IPA(M) and $\%IPA_{PRU}$ in citrate samples was 80.3 and 72.3 %, respectively, and, in napsagatran samples 97.5 and 97.6 %, respectively, all at 1 h post-dose.

3.5 Pharmacokinetic-Pharmacodynamic Relationship

The pharmacodynamic data collected during the clinical study alone were not informative enough to identify an I_{max}-type model (Eq. 3) for the pharmacokinetic-pharmacodynamic relationship. Thus, information from the in vitro IC_{50} measurements was incorporated in addition to the clinical data, and a Bayesian approach using weak prior information on the model parameters yielded model fits (Fig. 4; Table 2). The estimated IC_{50} for %IPA(M) and $\%IPA_{PRU}$ of citrate samples obtained from the pharmacokinetic-pharmacodynamic model (Eq. 3) were 41.5 and 48.0 pmol/mL, respectively.

The Spearman rank correlation coefficient between %IPA(M) and $\%IPA_{PRU}$ values using either peak

Table 1 Plasma pharmacokinetic variables of ACT-246475 in Part I and Part II

Part I	F1, 20 mg $N = 6$	F1, 80 mg $N = 6$	F1, 320 mg $N = 6$	F1, 1,000 mg $N = 6$
C_{max} [pmol/mL]	2.2 (1.1, 4.5)	4.0 (2.9, 5.7)	7.6 (4.4, 13.1)	13.8 (9.7, 19.5)
t_{max} [h]	1.8[a] (1.5–2.0)	1.8 (1.0–3.0)	3.0 (1.5–6.0)	4.5 (3.0–8.0)
AUC_{last} [h·pmol/mL]	2.0[a] (0.2, 17.9)	11.8 (8.5, 16.3)	38.8 (21.3, 70.8)	135.8 (104.5, 176.6)
$AUC_{0-\infty}$ [h·pmol/mL]	n.c.	18.6[b, d] (14.6, 23.7)	46.2[c, d] (22.7, 94.2)	193.8[c, d] (155.9, 241.1)
$t_{½}$ [h]	n.c.	2.4[b] (1.0, 5.5)	3.9[c] (1.7, 9.1)	9.9[c] (5.9, 16.5)
Part II	**F1, 70 mg $N = 9$**		**F2, 70 mg $N = 9$**	**dF, 50 mg $N = 9$**
C_{max} [pmol/mL]	4.7 (3.0, 7.2)		39.5 (30.4, 51.3)	10.3 (5.7, 18.7)
t_{max} [h]	2.0[c] (1.0–3.0)		1.0 (1.0–1.0)	1.0 (0.5–1.5)
AUC_{last} [h·pmol/mL]	10.2 (4.8, 21.9)		82.6 (64.4, 105.9)	22.9 (14.3, 36.6)
$AUC_{0-\infty}$ [h·pmol/mL]	26.4[c, d] (16.3, 42.6)		87.1 (68.2, 111.2)	39.1[d] (28.0, 54.5)
$t_{½}$ [h]	2.2[c] (1.2, 4.0)		1.3 (1.1, 1.6)	3.2 (1.6, 6.3)

Part I single oral doses of 20, 80, 320, and 1,000 mg ACT-281959 as F1, *Part II* single oral doses of 70 mg ACT–281959 as F1 and F2, and 50 mg ACT-246475 as dF, under fasted conditions, in healthy male subjects

C_{max} maximum plasma concentration, t_{max} time to reach maximum plasma concentration, AUC_{last} area under plasma concentration-time curve from zero to last measurable concentration, $AUC_{0-\infty}$ area under plasma concentration-time curve from time zero to infinity, $t_{½}$ terminal half-life, *n.c.* not calculated

Data are geometric mean (95 % CI) and median (range) for t_{max}

[a] $N = 4$, [b] $N = 3$, [c] $N = 5$, [d] AUC_{extra} represents more than 20 % of $AUC_{0-\infty}$ (3 subjects each within the 80, 320, and 1,000-mg group; 3 subjects after F1 and 6 subjects after dF)

Table 2 Pharmacokinetic-pharmacodynamic model parameter estimates for %IPA(M) and $\%IPA_{PRU}$ (citrate) using clinical data and method validation data (as model priors)

Parameter	% IPA(M)		$\%\ IPA_{PRU}$	
	Typical value	SD	Typical value	SD
I_{max}	87.6	5.4	94.0	4.2
IC_{50} (pmol/mL)	41.5	8.4	48.0	4.7
γ	1.29	0.13	0.96	0.07
$I_{placebo}$	−2.3	0.66	1.15	1.05
Resid. SE	10.0	0.37	8.6	0.3

I_{max} maximum inhibition, IC_{50} inhibitor concentration at half-maximal inhibition, γ shape/steepness factor, $I_{placebo}$ inhibition associated with placebo, *Resid. SE* residual standard error

%IPA(M) and $\%IPA_{PRU}$ (Eq. 2) values or all individual values, was 0.68 and 0.43 ($P < 0.001$), respectively, if subjects allocated to placebo treatment were excluded, and 0.66 and 0.40 ($P < 0.001$), if subjects allocated to placebo treatment were included.

4 Discussion

This Phase I study was the first trial in humans with ACT-245475 and the di-ester prodrug ACT-281959, designed to explore the safety, tolerability, pharmacokinetics, and pharmacodynamics in healthy males when administered as single doses of a formulation of the di-ester prodrug ACT-281959. The selection of healthy male subjects as study population was based on the consideration that at the time of this first trial in human subjects no reproductive toxicity studies had yet been performed. Preliminary preclinical investigations in rats and dogs resulted in a higher exposure (up to 2-fold) in female than in male animals. Clearance was investigated in female and male rats, and was slightly lower in the former. A dedicated gender study in human subjects is needed to explore any differences in pharmacokinetics, as well as in pharmacodynamics, and in the pharmacokinetic- pharmacodynamic relationship. In a recent study investigating the platelet response to single doses of ticagrelor in elderly and in young females, compared to young males, the authors reported higher platelet sensitivity to treatment in young males than in female and elderly subjects [28].

The effect of various formulations and of the double-prodrug on the pharmacokinetics of ACT-281959 and ACT-246475 had been investigated in rats and dogs and showed species differences. Similarly, preclinical data were not consistent in supporting dF would lead to a higher bioavailability in man than its prodrug. F1 was selected for Part I of this study because of the wide range of doses that could be administered which supported the study design. F1 is an

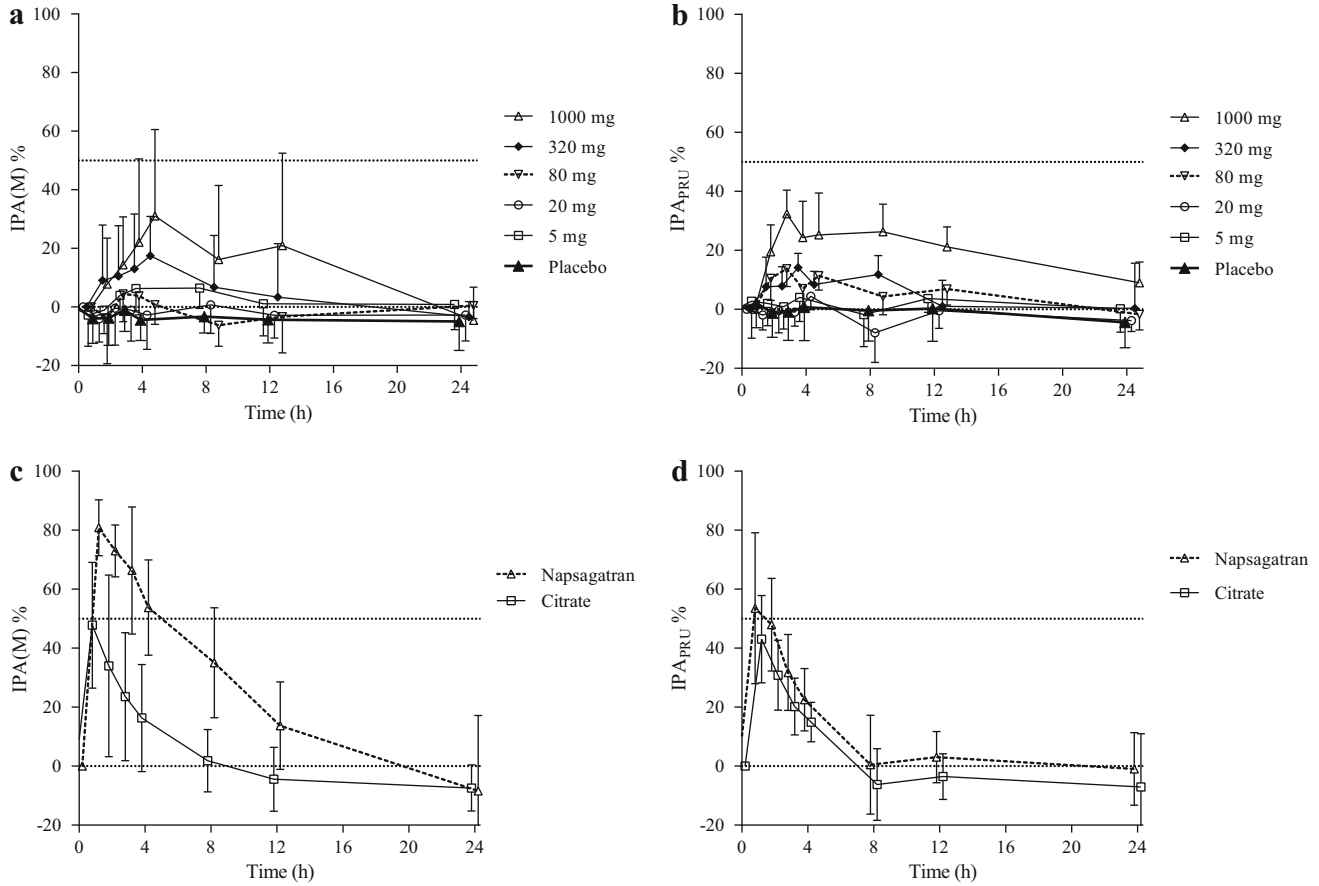

Fig. 3 Mean (SD) **a** %IPA(M)-time profiles and **b** %IPA$_{PRU}$-time profiles after single oral administration of 5, 20, 80, 320, and 1,000 mg ACT-281959 ($n = 6$) or placebo ($n = 10$) as F1 in Part I in healthy male subjects, under fasted conditions. Mean (SD) **c** %IPA(M)-time profiles and **d** %IPA$_{PRU}$-time profiles in citrate and napsagatran after single oral administration of F2 (70 mg ACT-281959, Part II), in healthy male subjects, under fasted conditions. Reference lines are displayed at $Y = 0$ %, 50 % (%IPA). *SD* Standard deviation, *%IPA* percent inhibition of platelet aggregation, *%IPA(M)* IPA calculated using MPA and Eq. 1, *%IPA$_{PRU}$* %IPA calculated using PRU and Eq. 1, *MPA* maximal platelet aggregation (LTA method), *PRU* device-derived $P2Y_{12}$ reaction units (VerifyNow $P2Y_{12}$ method)

emulsion liquid formulation, in which ACT-281959 is dissolved in the hydrophobic phase of the emulsion and it allowed administration of high doses of ACT-281959. A solution formulation (F2) of ACT-281959, i.e., a formulation in which aqueous and organic excipients are mixed in one phase and from which the drug is readily available to partition into aqueous gastrointestinal fluids, was developed to explore any effect of formulation on the oral absorption process. F2 is characterized by low palatability and thus a dose of ACT-281959 F2 of 80 mg or lower (i.e., total F2 volume of 200 mL or lower, ACT-281959 concentration in F2 0.4 mg/mL) could be selected in Part II. An ACT-281959 dose not yet tested in Part I was chosen for Part II (70 mg). In preclinical studies F2 showed up to 6-fold increase in exposure to ACT-246475 (rat studies), and, after having shown that ACT-281959 doses up to 1,000 mg of F1 were well tolerated (Part I), the 70 mg dose of ACT-281959 as F2 was considered suitable for testing in Part II.

All formulations, at all tested doses, were well tolerated. No relationship between doses or formulations and occurrence or intensity of AEs was observed. No SAEs, AEs of severe intensity, and no bleeding-related events or dyspnea were reported during the study.

Following single oral administration of up to 1,000 mg of ACT-281959 (F1) under fasted conditions, the increase in ACT-246475 C_{max} was less than dose-proportional, whereas AUCs increased proportionally with dose. Peak plasma concentrations were achieved later when higher F1 doses were tested, which resulted in a less than dose-proportional increase of C_{max} among doses. These could be due to a slower release of ACT-281959 from the emulsion formulation F1 at higher doses due to its low solubility in aqueous media, or to a saturable absorption process. No preclinical studies were yet conducted to identify potential transporters involved in the absorption from the gastrointestinal tract. However, the higher exposure to

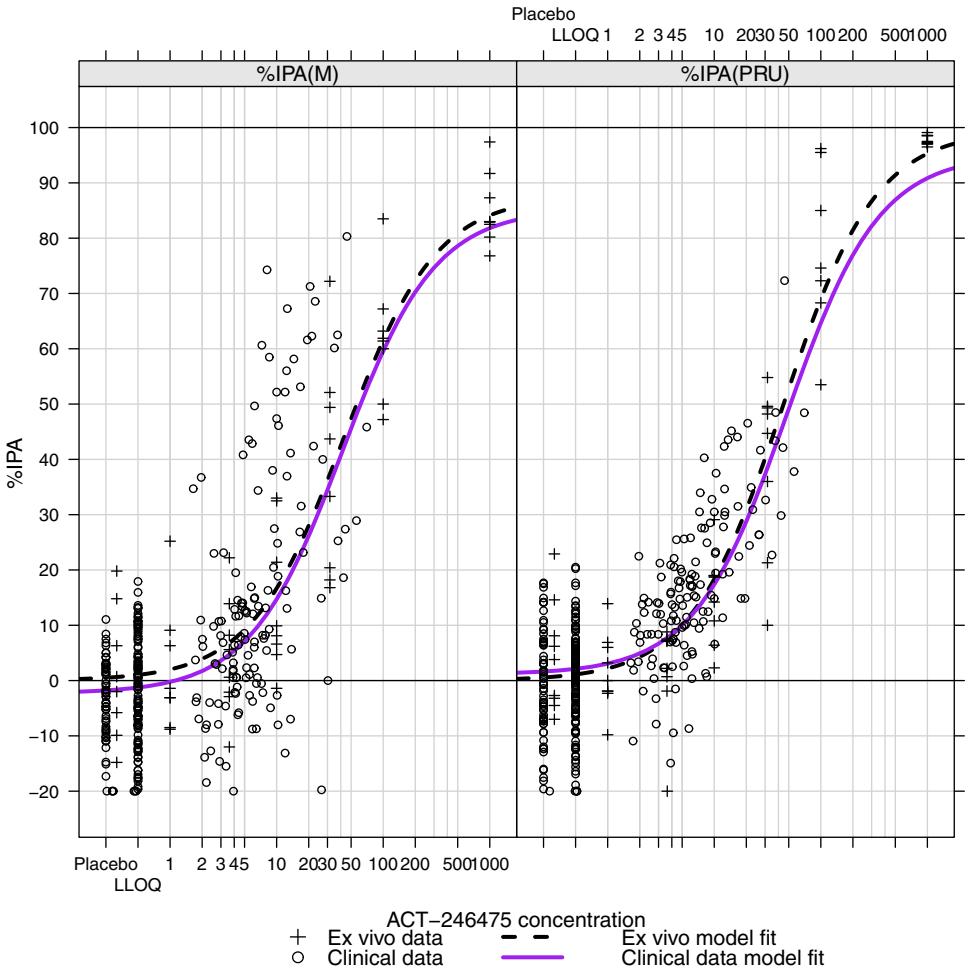

Fig. 4 %IPA(M) (*left panel*) and $\%IPA_{PRU}$ (*right panel*) in citrate versus ACT-246475 concentrations (semi-logarithmic scale) during assay validation and in the clinical study with I_{max} model fits. *%IPA* percent inhibition of platelet aggregation, *%IPA(M)* IPA calculated using MPA and Eq. 1, $\%IPA_{PRU}$ IPA calculated using PRU and Eq. 1, *MPA* maximal platelet aggregation (LTA method), *PRU* device-derived $P2Y_{12}$ reaction units (VerifyNow $P2Y_{12}$ method), I_{max} maximal inhibition calculated using Eq. 2

ACT-246475 observed after administration of F2 (70 mg ACT-281959) and the active moiety dF (50 mg ACT-246475) than after F1 (70 mg ACT-281959) suggests that ACT-281959 partition from F1 (emulsion formulation) to gastrointestinal fluids, rather than potential transporters, probably constitutes the limiting kinetic step. Thus, with increasing doses in Part I, the release of ACT-281959 from F1 resulted in sustained absorption: t_{max} occurred later and the $t_{½}$ was longer. The longest $t_{½}$ was seen in the 1,000 mg dose group and was between 2.5 and 7.6 fold longer than in all other dose/treatment groups in Part I and II. In Part II, absorption and elimination after F2 and dF were both fast, with t_{max} at 1 h and $t_{½}$ of 1.3–3.2 h.

Nonclinical studies provided evidence that hydrolysis of the di-ester and mono-ester prodrugs is rapid. Using a Caco-2 model [29] it appeared that ACT-281959 is partially hydrolyzed to yield mainly ACT-409100 and in smaller amounts ACT-246475, indicating that enterocytes may represent the initial site of hydrolysis. ACT-281959 is lipophilic with log $D > 5.8$ (octanol/phosphate buffer pH 7.4) whereas ACT-246475 is hydrophilic and mostly ionized at pH 7.4 (pKa = 2.1 and 6.8) with log $D = 0.0$.

Accordingly, systemic exposure to both ACT-281959 and the intermediate ACT-409100 during the study at all doses and for both F1 and F2, as well as to ACT-246475, was low.

Ex vivo monitoring of ADP-induced platelet aggregation was performed to explore pharmacokinetic-pharmacodynamic relationships. Two different assays for the measurement of ADP-induced platelet aggregation (LTA and VerifyNow $P2Y_{12}$) were used. Light transmission aggregometry is a gold standard in the exploratory characterization of $P2Y_{12}$ antagonists. However, the assay has several limitations: extensive and laborious sample preparation, requirement of trained technical staff, and high intrinsic variability [30, 31]. VerifyNow $P2Y_{12}$ is based on the same principles as LTA, but it does not require sample preparation or specific technical expertise and is highly reproducible. Previous studies have shown that %IPA derived by the VerifyNow $P2Y_{12}$ correlates with those derived by LTA [30, 31]. In this trial, both assays were tested. The in vitro method validation revealed good correlation, similar IC_{50} values (Eq. 3) were obtained with the two assays [42.7 pmol/mL for %IPA(M) and 41.9 pmol/mL for $\%IPA_{PRU}$ (Eq. 2)].

During the clinical study, at the tested doses and with the tested formulations, plasma concentrations of ACT-246475 remained below the IC_{50} determined in citrate. Consistently, in Part I the peak %IPA remained below 50 %, with the highest mean values seen after administration of the 1,000 mg ACT-281959 F1 dose (31.0 % [%IPA(M)] and 32.4 % [$\%IPA_{PRU}$]).

In Part II, pharmacodynamic measurements were performed only after administration of F2, using as anticoagulant citrate and napsagatran. Citrate is commonly used, in protocols for LTA and VerifyNow $P2Y_{12}$ assays, for anticoagulation of blood samples. However, by chelating calcium, citrate reduces the free calcium levels in the blood samples thus interfering with the platelet aggregation process. Previous studies have reported a potential underestimation of the %IPA of $P2Y_{12}$ inhibitors due to higher platelet aggregation values obtained in citrate than with other anticoagulants [25]. The selection of optimal doses of $P2Y_{12}$ inhibitors to be tested during clinical development is based on targeting %IPA values between 70 and 90 %. An underestimation of %IPA could thus lead to selecting a supratherapeutic dose, accompanied with higher risk of hemorrhagic side effects [32]. Therefore, in this study we tested the extent of platelet inhibition in napsagatran (a thrombin inhibitor) [26] and compared it with the standard assays performed with citrate. Napsagatran prevents blood coagulation without affecting the free calcium levels, thus better reflects physiological conditions than citrate, and might be of relevance for the dose selection process.

The pharmacodynamic assessment after administration of dF and F1 could not be performed because of limitations in the total blood volume that could be withdrawn from the subjects during the 3 treatment periods. The higher exposure obtained after administration of F2 resulted in up to approximately 10-fold higher peak %IPA(M) in citrate than after a similar dose of ACT-281959 as F1 in Part I (80 mg), whereas the increase in mean peak $\%IPA_{PRU}$ (citrate) was only 3-fold. This difference was due to the low values obtained with the two assays after F1 (after 80 mg ACT-281959 F1, %IPA(M) and $\%IPA_{PRU}$ were 4.5 and 13.7 %, respectively), whereas the mean peak %IPA after F2 was similar in both assays (mean peak of %IPA(M) and $\%IPA_{PRU}$ 47.8 and 43.1 %, respectively).

A substantial inter-subject variability was observed for ACT-246475-induced platelet inhibition as measured by both LTA assay and VerifyNow $P2Y_{12}$. However, these findings cannot be definitive due to the low sample size and the low %IPA values and ACT-246475 plasma concentrations measured during the clinical study. The assay precision at %IPA values of 10–20 % is only moderate. Consistently, the correlation observed between the two assays (citrate) for the clinical data was lower than during assay validation.

The use of an anticoagulant not interfering with calcium levels (napsagatran) was shown to affect pharmacodynamic measurements of ACT-246475. The IPA(M)% values (LTA assay) measured in blood samples collected in napsagatran were higher than in citrate samples, which was consistent with results observed during method validation. ACT-246475 C_{max} after administration of F2, i.e., 39.5 pmol/mL (geometric mean), was approximately 2.9 fold higher than the IC_{50} derived in vitro for %IPA(M) in napsagatran samples (13.8 pmol/mL), and fell below this threshold from 3 h post-dose. The mean peak %IPA(M) in napsagatran was 80.8 % (1 h post-dose) and remained above 50 % for approximately 6 h post-dose. Thus, the recovery in platelet ADP-induced aggregation in napsagatran was delayed compared to the plasma concentration-time profiles. The correlation of %IPA measured with LTA in napsagatran to clinical outcome is unknown and thus it should be prospectively explored.

Measurements in napsagatran were also performed with VerifyNow $P2Y_{12}$ during the clinical study. Differently from LTA, VerifyNow $P2Y_{12}$ measurements in napsagatran showed similar values of mean $\%IPA_{PRU}$ as samples in citrate, with SDs in the former nearly 2 fold higher. Replacement of citrate with napsagatran required manual addition of the anticoagulant in the sampling tubes, whereas the VerifyNow $P2Y_{12}$ apparatus best performs when vacuum is preserved in the blood collection tubes (manufacturer instructions). Thus, despite napsagatran possibly improves detection of platelet susceptibility to $P2Y_{12}$ antagonists with LTA, it may not be appropriate when using the VerifyNow $P2Y_{12}$ system.

5 Conclusions

Single oral doses of ACT-281959 and ACT-246475 were well tolerated in healthy male subjects. Inhibition of platelet aggregation correlated with ACT-246475 plasma concentrations. The systemic exposure to ACT-246475 could be enhanced by exploratory formulations. The development of new formulations or prodrug derivatives improving the systemic bioavailability of ACT-246475 is needed to support its further clinical development.

Acknowledgements The contributions of Ines Paule, PhD (Actelion Pharmaceuticals Ltd) for the design and execution of the pharmacokinetic-pharmacodynamic analysis, of Swiss Bioanalytics AG for the bioanalysis of pharmacokinetic samples, of Alexander Treiber, PhD (Actelion Pharmaceuticals Ltd) for the nonclinical (in vitro and in vivo) pharmacokinetic studies, and of Eva Caroff (Actelion Pharmaceuticals Ltd) for providing the chemical structures of the active and the prodrugs are acknowledged. The study was funded by Actelion Pharmaceuticals Ltd. D. Baldoni, S. Bruderer, M. Gutierrez, and J. Dingemanse contributed to the study design and the analysis and interpretation of the data. A. Krause contributed to the

pharmacokinetic-pharmacodynamic analysis and outputs; P. Gueret contributed to the setting and the validation of the pharmacodynamic methods and the analysis of the pharmacodynamic clinical data; B. Astruc was the Principal Investigator and was responsible for the conduct of the study and the collection and interpretation of the data. All authors contributed to the preparation and review of the manuscript and approved the final version for submission.

Conflict of interest The study was funded by Actelion Pharmaceuticals Ltd. D. Baldoni, S. Bruderer, A. Krause, M. Gutierrez, P. Gueret, B. Astruc, and J. Dingemanse had support from Actelion Pharmaceuticals Ltd for the submitted work; D. Baldoni, S. Bruderer, A. Krause, M. Gutierrez, and J. Dingemanse were full-time employees of Actelion Pharmaceuticals Ltd at the time of the study, B. Astruc is a full-time employee of Biotrial, which received funding from Actelion Pharmaceuticals Ltd, P. Gueret is a subcontracted consultant of Biotrial; no other relationships or activities that could appear to have influenced the submitted work. Authors have full control of all primary data and agree to allow the journal to review their data if requested.

Ethical standards The study was conducted in full conformity with the principles of the Declaration of Helsinki, the EMA Note for Guidance on Good Clinical Practice (CPMP/ICH/135/95), and the laws and regulations of France. The Ouest VI Committee for the Protection of Persons (Ethics Committee) located in Brest (France) approved the study protocol (approval number: CPP Ouest 6 - 701) and all subjects gave written informed consent after full explanation of the research involved and prior to any screening procedures.

References

1. Davies MJ, Thomas AC, Knapman PA, Hangartner JR. Intramyocardial platelet aggregation in patients with unstable angina suffering sudden ischemic cardiac death. Circulation. 1986;73:418–27.
2. Michelson AD. Antiplatelet therapies for the treatment of cardiovascular disease. Nat Rev Drug Discov. 2010;9:154–69.
3. Davi G, Patrono C. Platelet activation and atherothrombosis. N Engl J Med. 2007;357:2482–94.
4. Montalescot G. Platelet biology and implications for antiplatelet therapy in atherothrombotic disease. Clin Appl Thromb Hemost. 2011;17:371–80.
5. Antman EM, Anbe DT, Armstrong PW, Bates ER, Green LA, Hand M, et al. ACC/AHA guidelines for the management of patients with ST-elevation myocardial infarction: a report of the American College of Cardiology/American Heart Association Task Force on Practice Guidelines (Committee to Revise the 1999 Guidelines for the Management of Patients with Acute Myocardial Infarction). Circulation. 2004;110:e82–292.
6. Smith SC Jr, Allen J, Blair SN, Bonow RO, Brass LM, Fonarow GC, et al. AHA/ACC guidelines for secondary prevention for patients with coronary and other atherosclerotic vascular disease: 2006 update: endorsed by the National Heart, Lung, and Blood Institute. Circulation. 2006;113:2363–72.
7. Anderson JL, Adams CD, Antman EM, Bridges CR, Califf RM, Casey DE, et al. 2011 ACCF/AHA Focused Update Incorporated Into the ACC/AHA 2007 Guidelines for the Management of Patients With Unstable Angina/Non-ST-Elevation Myocardial Infarction: a report of the American College of Cardiology Foundation/American Heart Association Task Force on Practice Guidelines. Circulation. 2011;123:426–579.
8. Dorsam RT, Kunapuli SP. Central role of the $P2Y_{12}$ receptor in platelet activation. J Clin Invest. 2004;113:340–5.
9. Minno MN, Guida A, Camera M, Colli S, Minno GD, Tremoli E. Overcoming limitations of current antiplatelet drugs: a concerted effort for more profitable strategies of intervention. Ann Med. 2011;43:531–44.
10. Andre P, Delaney SM, LaRocca T, Vincent D, DeGuzman F, Jurek M, et al. $P2Y_{12}$ regulates platelet adhesion/activation, thrombus growth, and thrombus stability in injured arteries. J Clin Invest. 2003;112:398–406.
11. Wiviott SD, Braunwald E, McCabe CH, Montalescot G, Ruzyllo W, Gottlieb S, et al. Prasugrel versus clopidogrel in patients with acute coronary syndromes. N Engl J Med. 2007;357:2001–15.
12. Vivas D, Angiolillo DJ. Platelet $P2Y_{12}$ receptor inhibition: an update on clinical drug development. Am J Cardiovasc Drugs. 2010;10:217–26.
13. Jernberg T, Payne CD, Winters KJ, Darstein C, Brandt JT, Jakubowski JA, et al. Prasugrel achieves greater inhibition of platelet aggregation and a lower rate of non-responders compared with clopidogrel in aspirin-treated patients with stable coronary artery disease. Eur Heart J. 2006;27:1166–73.
14. Wallentin L, Varenhorst C, James S, Erlinge D, Braun OO, Jakubowski JA, et al. Prasugrel achieves greater and faster $P2Y_{12}$ receptor-mediated platelet inhibition than clopidogrel due to more efficient generation of its active metabolite in aspirin-treated patients with coronary artery disease. Eur Heart J. 2008;29:21–30.
15. Yusuf S, Zhao F, Mehta SR, Chrolavicius S, Tognoni G, Fox KK. Effects of clopidogrel in addition to aspirin in patients with acute coronary syndromes without ST-segment elevation. N Engl J Med. 2001;345:494–502.
16. Fox KA, Mehta SR, Peters R, Zhao F, Lakkis N, Gersh BJ, et al. Benefits and risks of the combination of clopidogrel and aspirin in patients undergoing surgical revascularization for non-ST-elevation acute coronary syndrome: the Clopidogrel in Unstable angina to prevent Recurrent ischemic Events (CURE) Trial. Circulation. 2004;110:1202–8.
17. Crouch MA, Colucci VJ, Howard PA, Spinler SA. $P2Y_{12}$ receptor inhibitors: integrating ticagrelor into the management of acute coronary syndrome. Ann Pharmacother. 2011;45:1151–6.
18. Caroff E, Meyer E, Treiber A, Hilpert K, Riederer MA. Optimization of 2-phenyl-pyrimidine-4-carboxamides towards potent, orally bioavailable and selective P2Y12 antagonists for inhibition of platelet aggregation. Bioorgan Med Chem Lett. 2014;24:4323–31.
19. Fung EN, Zheng N, Arnold ME, Zeng J. Effective screening approach to select esterase inhibitors used for stabilizing ester-containing prodrugs analyzed by LC-MS/MS. Bioanalysis. 2010;2:733–43.
20. FDA Guidance for Industry Bioanalytical Method Validation. 2001; Available from: 2001 May. http://www.fda.gov/downloads/Drugs/…/Guidances/ucm070107.pdf. Accessed 10 Jul 2011.
21. EMA. Guideline on Validation of Bioanalytical Methods 2009; Available from 15 Nov 2009. http://www.ema.europa.eu/docs/en_GB/document_library/Scientific_guideline/2009/12/WC500018062.pdf. Accessed 10 Jul 2011.
22. Gough K, Hutschinson M, Keene O, et al. Assessment of dose proportionality: report from the statisticians in the pharmaceutical industry/pharmacokinetics UK joint working party. Drug Inform J 1995;29:1039–48.
23. Julious SA, Debarnot CA. Why are pharmacokinetic data summarized by arithmetic means? J Biopharm Stat. 2000;10:55–71.
24. Michelson AD. Methods for the measurement of platelet function. Am J Cardiol. 2009;103:20A–6A.
25. Pittens CA, Bouman HJ, van Werkum JW, ten Berg JM, Hackeng CM. Comparison between hirudin and citrate in monitoring the inhibitory effects of $P2Y_{12}$ receptor antagonists with different platelet function tests. J Thromb Haemost. 2009;7:1929–32.

26. Gast A, Tschopp TB, Schmid G, Hilpert K, Ackermann J. Inhibition of clot-bound and free (fluid-phase thrombin) by a novel synthetic thrombin inhibitor (Ro 46-6240), recombinant hirudin and heparin in human plasma. Blood Coagul Fibrinolysis. 1994;5:879–87.
27. Lunn DJTA, Best N, Spiegelhalter D. WinBUGS—a Bayesian modelling framework: concepts, structure, and extensibility. Stat Comput. 2000;10:325–37.
28. Teng R, Mitchell P, Butler K. Effect of age and gender on pharmacokinetics and pharmacodynamics of a single ticagrelor dose in healthy individuals. Eur J Clin Pharmacol. 2012;68:1175–82.
29. Meunier V, Bourrie M, Berger Y, Fabre G. The human intestinal epithelial cell line Caco-2; pharmacological and pharmacokinetic applications. Cell Biol Toxicol. 1995;11:187–94.
30. Breet NJ, van Werkum JW, Bouman HJ, Kelder JC, Ruven HJ, Bal ET, et al. Comparison of platelet function tests in predicting clinical outcome in patients undergoing coronary stent implantation. JAMA. 2010;303:754–62.
31. Gremmel T, Steiner S, Seidinger D, Koppensteiner R, Panzer S, Kopp CW. Comparison of methods to evaluate clopidogrel-mediated platelet inhibition after percutaneous intervention with stent implantation. Thromb Haemost. 2009;101:333–9.
32. Michelson AD, Frelinger AL 3rd, Furman MI. Current options in platelet function testing. Am J Cardiol. 2006;98:4N–10N.